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The Editor comments

IN THE INTEREST OF BETTER SUSPENSIONS

In this issue we are carrying four papers which discuss modern approaches, aims, and concepts of pharmaceutical suspension formulation. The authors describe and discuss old problems which have been overcome, and new problems which have evolved as a result of the use of new techniques and agents coupled with the demand for increased pharmaceutical elegance and efficacy.

These papers were originally presented in the form of a symposium during the first annual meeting of the newly created A. PH. A. Section on Industrial Pharmacy. The Section officers and program committee are to be complimented on the excellent choice of individuals who were selected for that program.

The preparation of satisfactory suspensions is one of the oldest problems in the history of pharmacy. And yet we only have to think back a few years to recall many suspension formulations which rapidly settled out, sometimes to the point of caking with the subsequent formation of cement-like precipitates. By applying basic approaches of chemistry and physics, this problem has been largely overcome in recent years. This achievement, while perhaps less dramatic, compares with that of the Wright brothers at Kitty Hawk—for both represent means of overcoming the basic law of gravity. In each case a relatively heavy object is held suspended in a medium of low density.

We feel, therefore, that this subject has very unusual significance. It represents a classic example of where the old and new of pharmacy have been effectively bridged by proper application and use of fundamental principles derived from the basic sciences.

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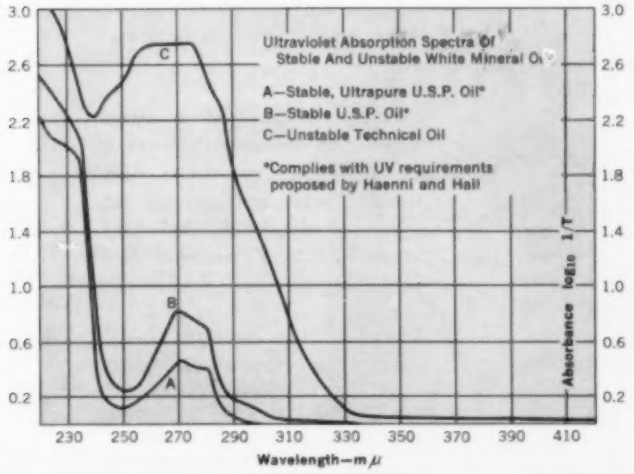

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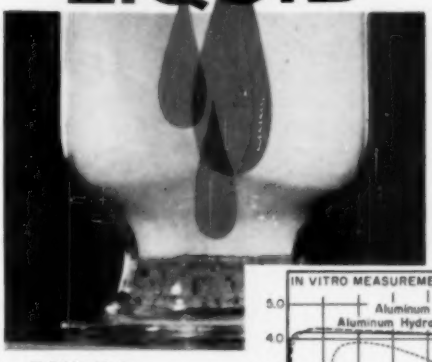

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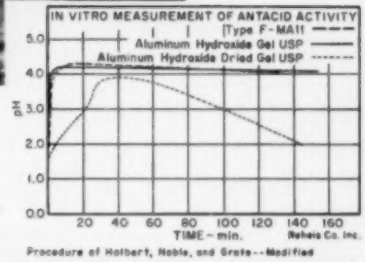

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Review Article

Application of Infrared Spectrophotometry to Pharmaceutical Analysis

By JONAS CAROL

In 1905, Cob'entz (1) pioneered the development of infrared spectrometry and showed clearly the great possibilities of this technique as an analytical tool. The complexity and instability of his equipment, however, precluded its use except under the most carefully controlled conditions. Forty years passed before advances in optics and electronics resulted in the production of infrared spectrophotometers suitable for general laboratory use. Then, with the impetus generated by World War II research demands, great strides were made in this field. Outstanding work was done in the studies made on petroleum fuels, synthetic rubber, and penicillin. At the close of the war, commercia infrared spectrophotometers became ava lable for nonmilitary use. Laboratories in almost every field of chemistry began investigating their utility.

The analysis of pharmaceutical preparations by infrared spectrophotometry offers many advantages unavailable to conventional methods. Practically all pharmaceuticals have distinct infrared spectra, and complex mixtures may often be analyzed by this technique without prior separation into component parts. Despite these obvious advantages, infrared spectrophotometry was not immediately accepted by laboratories engaged in pharmaceutical analysis. The high cost of equipment, difficulties of sample-handling, scarcity of personnel skilled in this technique, and reluctance to change appeared to be factors causing this neglect. Gradually, all objections to its use have been overcome; today these instruments are an indispensable part of every drug laboratory. The inclusion of many infrared tests in the U.S.P. XVI (2) and the N. F. XI (3) makes possession of these instruments a necessity or both commercial pharmaceutical manufacturers and regulatory agencies. The Federal Food and Drug Administration has modern infrared spectrophotometers in every laboratory involved in drug analysis.

Received from the Department of Health, Education, and Welfare, Food and Drug Administration, Bureau of Biological and Physical Sciences, Division of Pharmaceutical Chemistry, Washington 25, D. C.

The author wishes to express his appreciation to his coworkers, Mrs. Alma L. Hayden and Mr. Oscar R. Sammul, for their help and advice in preparing this manuscript.

THEORETICAL PRINCIPLES

The absorbance of infrared radiation is a phenomenon associated with energy changes at the atomic level within molecules. The stretching and bending of bonds between atoms, and the rotation of the molecule as a whole, occur at frequencies ranging from 10^{12} to 10^{14} cycles/sec. When radiation of these frequencies is passed through a substance these modes of vibration and rotation are excited; energy is transferred from the radiant beam to the molecule. A measure of this energy transfer, relative to wave-

length or frequency, is the infrared absorption spectrum. The modes of vibration of a nonlinear molecule are given by the equation $3n - 6$, where n = number of atoms within the molecule. Theoretically, each of these modes results in a region of absorbance. The infrared spectra of even simple molecules are complex.

No two compounds, with the exception of enantiomorphs, have identical infrared absorption spectra. Slight differences in structure usually result in marked differences in spectra. It is usually easy to distinguish *ortho*, *meta*, and *para* isomers or *cis* and *trans* isomers and to identify each. This property of the infrared spectrum accounts for its great usefulness as a qualitative tool. The relationship between the infrared spectra and structures of organic compounds has been exhaustively investigated and documented (4–6). The location of certain bands has been calculated (and verified) for simple molecules. In most cases, however, these relationships have been found by empirical methods. The wavelengths at which absorbances occur, due to functional groups such as hydroxyl, amino, carbonyl, carbalkoxyl, alkoxy, etc., are not drastically shifted by structural changes in the molecule, however, spectral bands due to the complex skeletal portions of the molecule are not so easily characterized.

The infrared absorption spectrum of a substance yields many clues regarding its structure. In general all other physical measurements merely confirm or refute its identity. In addition to its utility in studies on the elucidation of structure, infrared spectrophotometry is invaluable for both qualitative and quantitative analysis.

INSTRUMENTATION

The first widely used commercial infrared spectrophotometers were single beam instruments using a direct current amplifier and a strip chart recorder. The spectrum recorded by these instruments represented the sum of the absorbance of sample, solvent, cell windows, and atmosphere. Disturbing zero drift was experienced in even the most carefully air-conditioned room. In addition, the lack of automatic slit control resulted in the characteristic "saw-tooth" spectrum due to the diminution of radiant energy as the spectrum was scanned from short to long wavelength. In spite of these difficulties, quantitative measurements could be made at a single wavelength by the "cell out-cell in" technique. In this technique, the reference cell, filled with solvent, was placed in the spectrophotometer beam with the instrument set at the desired wavelength, the shutter was closed and the instrument adjusted to read zero transmittance. The shutter was then opened and the slit was adjusted to produce 100 per cent transmittance. The reference cell was then replaced by the sample cell, and transmittance of the sample read directly from the recorder. However, the preparation of a qualitative spectrum from 2 to 15 μ required patience and considerable drafting ability. Improvements in instrumentation soon eased these difficulties. Plyler and co-workers (7) constructed a gear-box slit control mechanism for the Perkin-Elmer model 12 B infrared spectrometer. This device opened the slit gradually with increasing wavelength to produce an approximately constant energy level throughout the spectrum. Similar commercial equipment soon followed. The introduction of the chopped beam and an alternating current amplifier that responded only to the frequency of the interrupted beam, eliminated zero drift due to external temperature changes. Interchangeable prisms and windows of halides other than sodium chloride extended the useful spectral range to 40 μ and provided greater resolution to the 2 to 9 μ region.

Double-beam direct recording infrared spectrophotometers, employing the null principle, became available in early 1950. These instruments record spectra directly and eliminate interference from atmospheric carbon dioxide and water vapor. Solvent absorbance is compensated as long as the reference channel transmits over 50 per cent of the incident energy. Although these spectrometers were quite costly, their utility and flexibility enabled them to replace the cheaper single-beam instruments.

Recently, inexpensive double-beam infrared spectrophotometers have been marketed by several commercial manufacturers. These small, rugged, and easily operated instruments have been accepted by laboratories engaged in pharmaceutical analysis. While they lack the versatility of the larger units, they are entirely satisfactory for most routine operations.

Improvements and new developments have constantly extended the usefulness of these double-beam instruments. Ordinate expansion, which provides for the magnification of absorbance up to 20× without materially increasing "noise," is available for the more expensive instruments, and enables the estimation of microgram amounts of sample. Beam-condensing systems can be used to record the spectrum of single fibers. Prism-grating combinations have provided the high degree of resolution especially needed for the study of the relationship of spectra to structures.

APPLICATIONS

Extensive reviews of the infrared literature have been made by Gore (8) annually from 1950 to 1954, and biennually since 1954. Bibliographies of the infrared spectra of steroids have been published by Dobriner, *et al.* (4), and Roberts, *et al.* (5). Recent compilations by Bellamy (6) and Jones and Sandorfy (9) systematically presented the relationships between structures and spectra. Many of these references relate to pharmaceuticals. Cänback (10) restricted his review to the infrared analysis of drugs.

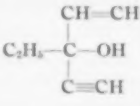
In this review, no attempt will be made to cover the literature completely; instead a number of carefully selected examples of representative procedures will be critically examined. To achieve uniform presentation, applications of infrared spectrophotometry to pharmaceutical analysis will be considered on a sample-handling basis rather than in chronological order. Initially these instruments were obtained by a few pharmaceutical laboratories in the hope of solving very difficult analytical problems for which conventional methods had failed. These early uses employed involved techniques; much of the later work is of a less complex nature.

Sample-handling in infrared spectrophotometry presents many difficulties not encountered in spectrophotometry in the visible and ultraviolet regions of the spectrum. These difficulties arise from limitations of both cell window material and solvents. Glass and quartz are opaque to wavelengths beyond about 3 μ. The windows usually employed, sodium chloride or potassium bromide, must be carefully protected from moisture. Other halides such as calcium fluoride, lithium fluoride, cesium bromide, silver chloride, and thallium brom-iodide (KRS 5) have limited uses due to other disadvantages. Recently a synthetic substance, Irtran-2,[1] has been developed that transmits radiation to 14 μ and is unaffected by water and most other solvents. Only carbon disulfide and carbon tetrachloride are satisfactory solvents, in thickness up to several millimeters, throughout the 2 to 15 μ spectral region. Chloroform and tetrachloroethylene are useful for measurements between 6 and 7 μ. A number of other solvents have "windows" in very limited regions or may be used with cells of 0.1 mm. thickness or less. Water and duterium oxide, in thin films, and with cell windows of silver chloride have had some use.

Limited solubility necessitates the use of solid-phase sample-handling for many substances. Sample preparations by this technique are more difficult to duplicate than in the case of solutions and quantitative analyses require careful attention to detail. The spectra of solids do have several advantages over those of solutions. The peaks in general are sharper and have more detail. The spectra of racemates (solid phase) are frequently different from either the *d*- or *l*- isomers due to compound formation.

The most commonly employed procedures can be divided into the following categories: films of liquids or solids; solutions in carbon disulfide, carbon tetrachloride, chloroform, etc., or derivatives (of insoluble substances) that dissolve in these solvents; dispersions in lquid petrolatum, in carbon disulfide, and in solid halides; gases.

Films.—A film of liquid between two halide windows is, perhaps, the simplest sample-handling device used in infrared spectrophotometry. The thickness of a film required to produce a satisfactory spectrum is controlled by varying the pressure applied to the windows. When a definite thickness of film is desired, metal foil spacers are employed. This method is strictly qualitative as it is impractical to measure accurately the thickness of the films normally used (0.025–0.100 mm.). Alcohol, glycerin, propylene glycol, paraldehyde, and polyethylene glycol 400 are typical of liquids included in the U. S. P. XVI. In this laboratory, the infrared spectrum of a film of β-chlorovinyl ethylethynyl carbinol, a mild hypnotic,



has been used for teaching purposes to demonstrate several interesting relationships between structure and spectra. This compound exhibits strong bands due to ≡C—H and =CHCl absorption in addition to the characteristic OH, CH, and C—Cl absorptions.

Furchgott and his co-workers (11) made the first detailed study of the infrared absorption spectra of the steroid hormones. Their work dealt mainly with the relationship of spectra to structure and was of a purely qualitative nature. Spectra were made from films of the steroids. The films were prepared by dissolving the compounds in a suitable organic solvent and adding the solution dropwise onto a heated sodium chloride plate. In most cases, pyridine was the solvent of choice. Heat-stable compounds were melted and allowed to solidify on the plate. Amorphous or glassy deposits were preferred over fine crystalline films since the latter resulted in excessive light scattering and strong back-

[1] Manufactured by Eastman Kodak Co., Rochester, N. Y.

ground. This background absorption was compensated to a considerable degree however, by use of a roughened salt plate in the reference beam of the spectrophotometer. A large number of compounds were examined, of which the following could be considered common pharmaceuticals: testosterone, testosterone propionate, estrone, estradiol-17β, estradiol benzoate, diethylstilbesterol, progesterone, cortisone, and desoxycorticosterone acetate.

The spectrum of a solid is influenced to a considerable degree by crystal size, form, and orientation (12). All of these factors are difficult to control in deposited films. In many cases it is almost impossible to produce a uniform deposit by the evaporation of solutions on a salt plate. The film tends to be thick on its periphery and thin in the center. For these reasons this sample-handling procedure is now rarely used, having been replaced by superior techniques.

In 1952, Newburger, *et al.* (13), described a technique for obtaining the infrared spectra of films of water-soluble gums. Although the procedure was primarily intended for the identification of gums in food products, it is also applicable to the analysis of pharmaceuticals. In this method a film of gum is deposited from aqueous solution onto a glass plate coated with water-repellent silicone. After drying, the film is removed with forceps, placed between salt plates, and its spectrum recorded from 2 to 15 μ. The spectra of acacia and tragacanth were among the fifteen gums and related products reported. They showed a general similarity, but were sufficiently different to enable positive identification of the individual gums. This method offers possibilities for the qualitative analysis of any water-soluble drug that can be deposited as an amorphous film.

Solutions.—The simplest and most accurate quantitative infrared analyses are possible where conventional spectrophotometric techniques can be applied; i. e., the measurement of absorbance of solutions of definite concentration using cells of known or compensated thickness. Analysis of single or multicomponent systems can be made by direct absorbance or baseline absorbance measurements provided a suitable solvent is available.

Many qualitative and quantitative methods for pharmaceuticals have been reported which employ solvent techniques. The U. S. P. XVI (page 925) and the N. F. XI (page 434) contain an infrared test for organic nitrogenous bases designed for the identification of antihistiminics. The base is precipitated from a solution of the salt with sodium hydroxide and extracted with carbon disulfide. The spectrum is recorded from 7 to 15 μ using 1-mm. cells and compared with a reference standard prepared in a similar manner. Eleven antihistiminics are listed in the two compendia. The spectra of a representative number of these shown in Fig. 1 illustrates the ease and certainty of identification of these compounds. This procedure would be applicable to any other carbon disulfide soluble base.

The determination of nitroglycerin in tablets (14) illustrates the convenience and specificity of many infrared spectrophotometric methods compared to conventional procedures. The present U. S. P. XVI assay (6) for nitroglycerin is based on the reduction of nitrate groups to ammonia, followed by distillation and titration with 0.01 *N* acid. This method is time-consuming, nonspecific, and with some samples gives erratic results.

Nitroglycerin is transparent to visible and ultraviolet light but has a distinctive infrared absorption spectrum with several strong maxima. In the infrared procedure, nitroglycerin is extracted from the powdered tablet sample with carbon disulfide. The extract is concentrated by evaporation and diluted to a definite volume to contain about 3 mg./ml. A standard solution is prepared from a nitroglycerin absorbate[2] in the same manner. The concentration of nitroglycerin in the tablet sample is calculated from the baseline absorbance of the sample and standard solutions measured at 7.89 μ. The identity is established by comparison of the spectra of sample and standard between 2 to 15 μ. The accuracy of the method was established by a collaborative study (15).

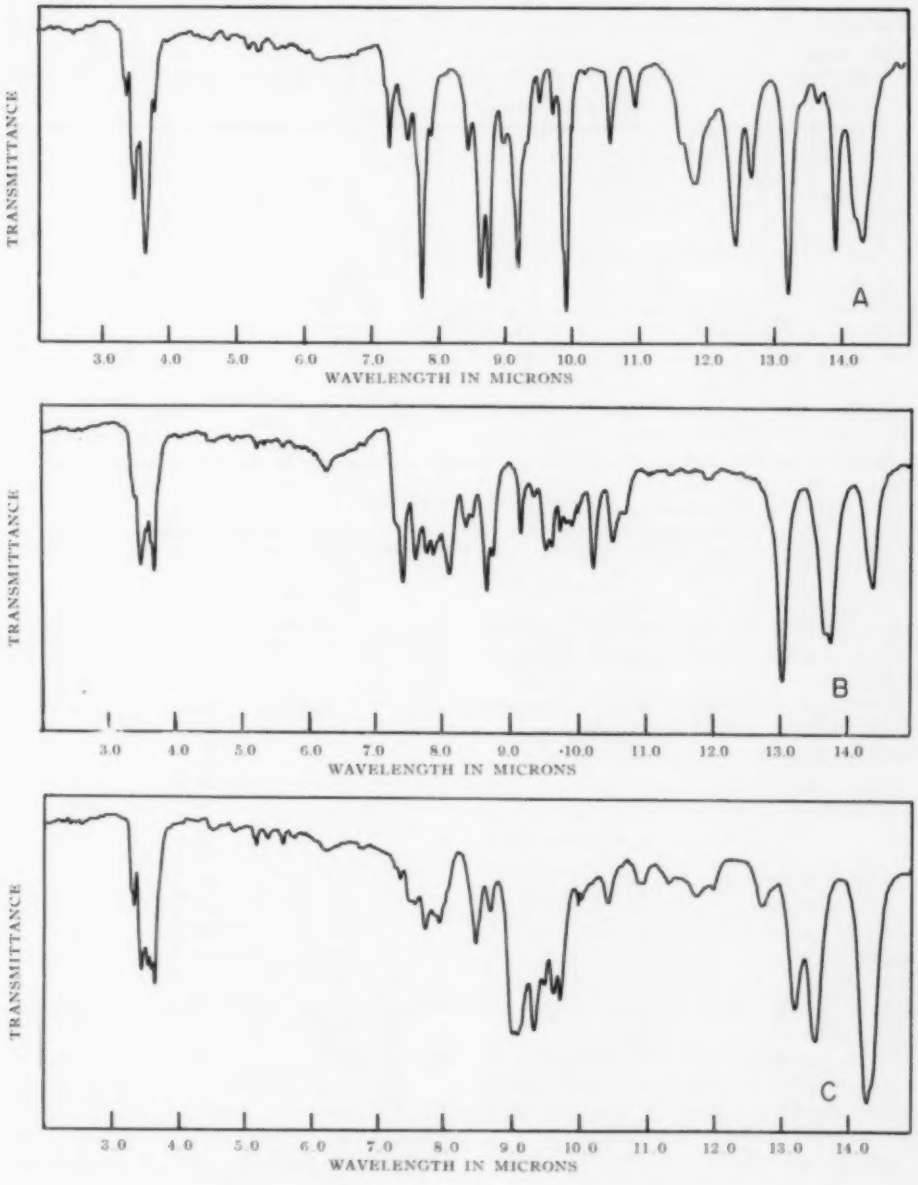
A committee of the Contact Section of the Pharmaceutical Manufacturers Association (16) has made a comprehensive study of this procedure with the intention of recommending its adoption as the U. S. P. method. A comparison of the results obtained by the U. S. P. assay procedure and the infrared method is shown in Table I. The Committee concluded: (*a*) The infrared method is more accurate and more specific than the present U. S. P. method. (*b*) The infrared method results in assay yields that are closer to theoretical than the present U. S. P. method. (*c*) The infrared method is simpler and more rapid than the U. S. P. method.

The infrared spectrophotometric analysis of atropine in pharmaceuticals (17), using the conventional carbon disulfide solution technique, has been reported. The accuracy and specificity

[2] Nitroglycerin can be obtained as a 10% absorbate on lactose. This material is stable for an indefinite period if kept tightly stoppered. It is standardized by the U. S. P. XVI assay.

of the method were established by a collaborative study. Equally satisfactory results have been obtained for hyoscine, codeine, nicotine, quinine, and quinidine.

This laboratory is occasionally required to analyze individual pharmaceutical tablets. Those of atropine, hyoscine, or a combination of the two may contain 0.3 mg. or less of total alkaloid. Analysis is made by the above macro procedure except that the alkaloidal residue is dissolved in 0.5 to 1.0 ml. of carbon disulfide and baseline absorbances of sample and standard solutions are measured using 5× ordinate expansion (18). Mixtures of the two alkaloids are separated chromatographically (19) prior to spectrophotometric est mation. The spectra of the two compounds at normal and 5× ordinate expansion are shown (Fig. 2) in the 9 to 12 μ


TRANSMITTANCE
A
3.0 4.0 5.0 6.0 7.0 8.0 9.0 10.0 11.0 12.0 13.0 14.0
WAVELENGTH IN MICRONS
TRANSMITTANCE
B
3.0 4.0 5.0 6.0 7.0 8.0 9.0 10.0 11.0 12.0 13.0 14.0
WAVELENGTH IN MICRONS
TRANSMITTANCE
C
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WAVELENGTH IN MICRONS

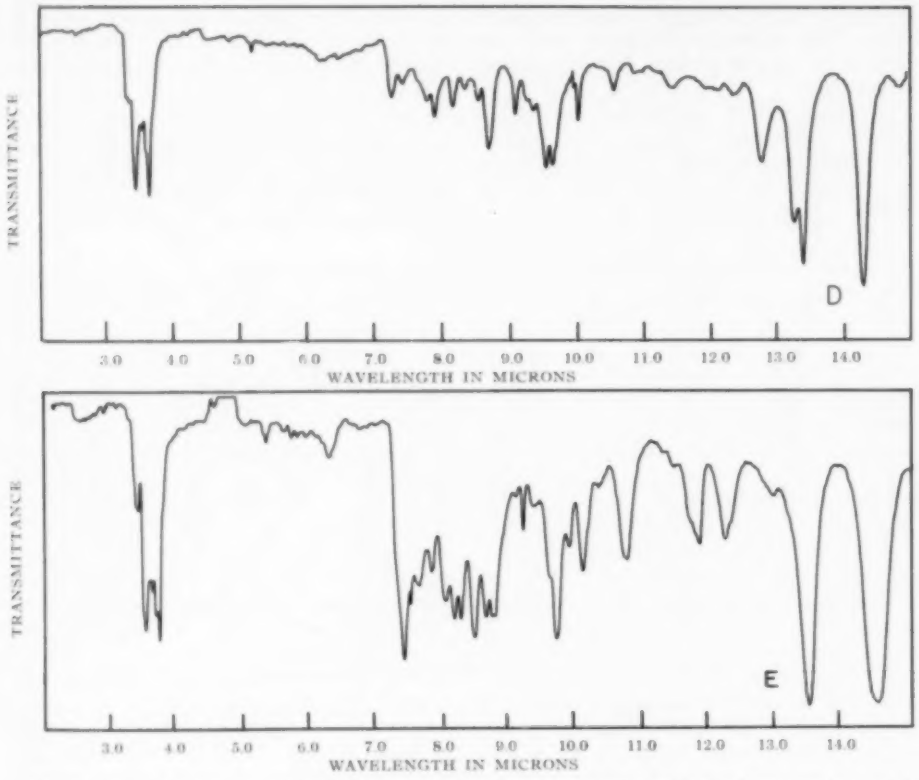

TRANSMITTANCE
D
3.0 4.0 5.0 6.0 7.0 8.0 9.0 10.0 11.0 12.0 13.0 14.0
WAVELENGTH IN MICRONS
TRANSMITTANCE
E
3.0 4.0 5.0 6.0 7.0 8.0 9.0 10.0 11.0 12.0 13.0 14.0
WAVELENGTH IN MICRONS

Fig. 1.—The infrared absorption spectra of antihistamines in carbon disulfide. Concentration, *ca.* 10 ml./mm. Cells 1.0 mm. (*A*) chlorcyclizine, (*B*) pyribenzamine, (*C*) diphenhydramine, (*D*) pheniramine, (*E*) methaphenilene.

region. Analysis of commercial tablet samples showed excellent recoveries based on labeled declarations.

Acceptance of quantitative spectrophotometric assays for drugs by official compendia is illustrated by the method for diethyltoluamide;

an arthropod repellant (U. S. P. XVI, p. 219). A specific estimation of the *meta* isomer is obtained by a modification of the carbon disulfide solution technique. The difference in absorbances ($A_{14.1\ \mu} - A_{14.5\ \mu}$) of sample and standard solutions is used instead of measurements at a single wavelength.

Many pharmaceuticals are practically insoluble in carbon disulfide, but are soluble in chloroform. The latter solvent is used in quantitative infrared analysis despite its relative opacity in some regions of the spectrum. Where solubility permits, cell thicknesses of 0.1 mm. or less are employed and the spectrum can be scanned from 2 to 15 μ. For substances of low solubility (1 mg./ml. or less), 1.0 mm. or thicker cells are required to obtain satisfactory absorbances. Under these conditions, quantitative measurements must be made at wavelengths

Table I. Comparison of Results of U.S.P. Assay Procedure and Infrared Method

Dosage, mg.	Sample	Nitroglycerin Found, % Infrared	U. S. P.
0.3	1	98.2	98.0
	2	99.0	97.3
	3	100.9	99.7
	4	98.8	99.0
0.4	1	98.8	92.5
	2	100.2	98.4
	3	98.8	95.0
	4	99.4	95.0
0.6	1	100.0	108.0
	2	98.9	104.2
	3	99.2	99.0
	4	100.1	99.2

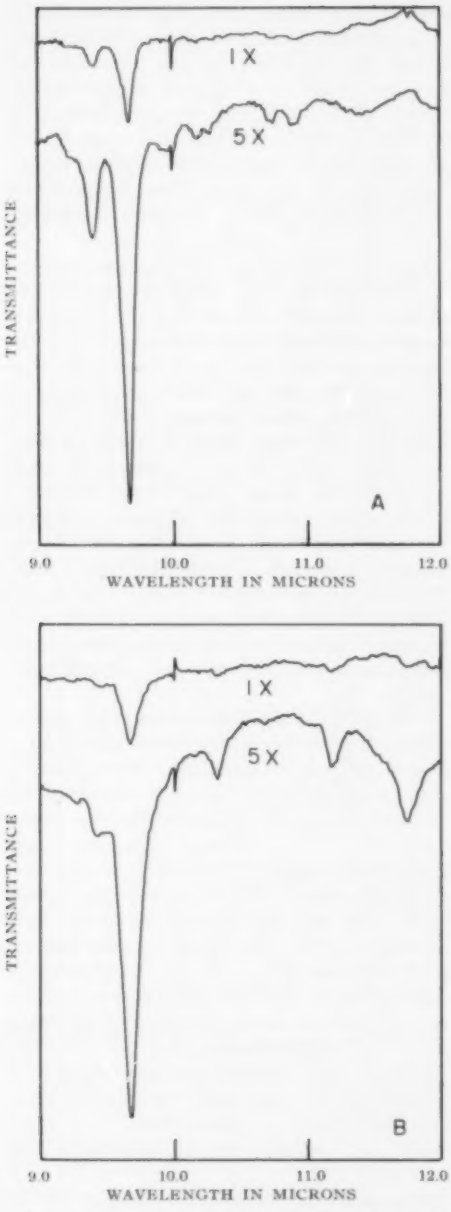


Fig. 2.—The infrared absorption spectra (10–13 μ) of (*A*) atropine and (*B*) hyoscine showing the effect of 5× ordinate absorption. Four tenths milligram per ml. in 1.0-mm. cells.

below 12 μ. Identification is then best accomplished using other sample-handling techniques.

The determination of the nitrate esters, erythritol tetranitrate, pentaerythritol tetranitrate, and mannitol hexanitrate is an example of the latter procedure (20). These esters are sparingly soluble in chloroform and analysis is made with 1-mm. cells. The conventional solvent technique is employed with absorbance measurements of sample and standard solutions at 6.0 μ. Identification is made by the potassium bromide disk technique.

Morris and his co-workers (21) described an infrared procedure for vitamins D_2 and D_3 based on the original observation of Jones (22) that the spectra of these substances differed at 10.3 μ. Both of the compounds are present in vitamin D from natural sources, and they are equally effective in the prevention and cure of rickets in mammals. In poultry, however, vitamin D_2 has practically no antirachitic activity. Therefore it was necessary to develop methods capable of estimating the relative amounts of the vitamin D forms in preparations intended for poultry use.

In the method, the total D vitamins were isolated from dosage forms by conventional procedures and determined colorimetrically on an aliquot of the prepared solution. The spectrum of a carbon disulfide solution of another aliquot was recorded from 10 to 11 μ using 1-mm. cells holding 0.25 ml. The relative amounts of the two forms were approximated by inspection (Fig. 3) or estimated (plus or minus 15 per cent) by the ratio $(A_{10.3\ \mu} - A_{10.5\ \mu})/(A_{10.4\ \mu} - A_{10.5\ \mu})$. Low potency feed supplement samples presented difficult extraction problems and final sample solutions usually contained substances contributing excessive background absorption. Such samples were assayed using a differential spectrophotometric technique in which D_2 or D_3 was added to the reference cell to compensate for absorbance due to this vitamin. The amount of vitamin D_2 or D_3 required indicated the form and quantity of vitamin D in the sample.

Washburn and Krueger (23) in 1949 used infrared spectrophotometry to assay acetylsalicylic acid, acetophenetidin, and caffeine mixtures without separating the component compounds. Analysis of this combination by the chemical means then available was tedious and time consuming. Analysis was achieved through the standard spectrophotometric technique for multicomponent systems in which absorbance measurements are made at a strong band for each component. At each of these bands there must be weak absorbance for the other components. The preparations of the sample solution require only simple chloroform extraction of the powdered tablet suspended in water. Absorbance measurements were made on sample and standard solutions at 9.27 μ (acetylsalicylic acid), 8.99 μ

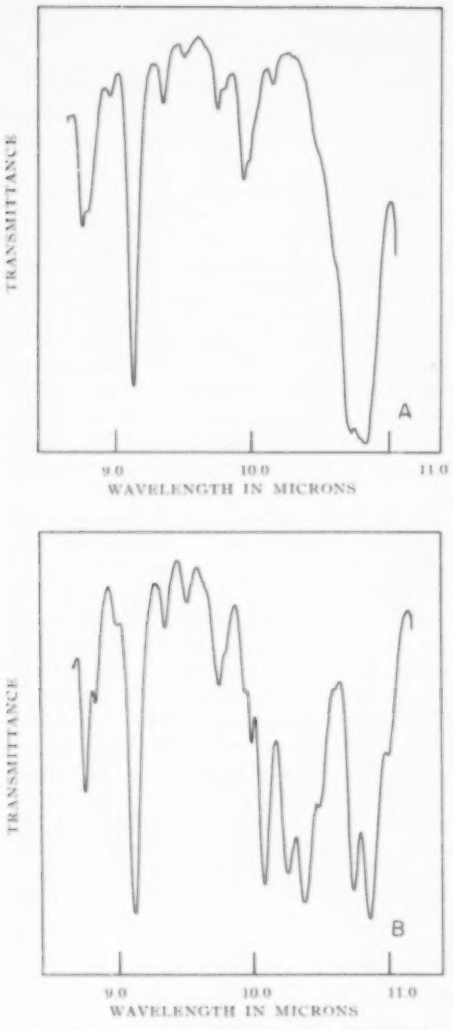


Fig. 3.—The infrared absorption spectra (10 μ region) of estradiol benzenesulfonyl esters conc. *ca.* 5 mg./ml. in 1.0-mm. cells (*A*) estradiol-17α (*trans*), (*B*) estradiol-17β (*cis*).

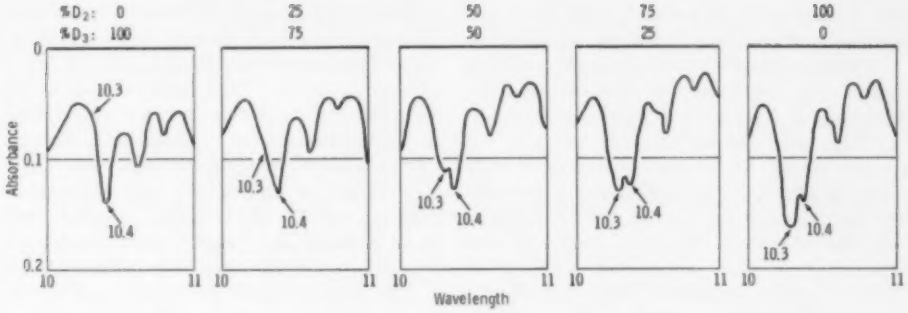
(acetophenetidin), and 10.26 μ (caffeine). Results were computed by approximation. The authors reported excellent recoveries of each compound from commerical tablet and capsule preparations. The findings were confirmed by a thorough collaborative study in 16 laboratories representing a significant segment of the pharmaceutical industry (24).

Infrared spectrophotometric equipment was first secured by this laboratory for the analysis of estrogenic hormones from natural sources. These estrogens were derived commercially from pregnant mares' urine and consisted chiefly of a complex mixture of the ketosteroids and related diols of widely varying therapeutic activity (see Chart I). Artifacts formed during the extraction process (25) further complicated the analytical problem. No chemical methods were available for the estimation of each physiologically active estrogen in these mixtures, and biological assays gave no indication of composition.

Isolation of the estrogens from impurities was accomplished by means of immiscible solvents. Further separation into ketonic and nonketonic fractions was made with Girard reagent T. The phenolic estrogens are practically insoluble in carbon disulfide, but are readily converted quantitatively to soluble benzene sulfonyl esters. A method for the three ketosteroids was described (26) using carbon disulfide solutions of these esters and the standard multicomponent spectrophotometric analytical technique. Absorbance measurements of sample and standard solutions (*ca.* 5 mg./ml., 1.0-mm. cells) were made at the three selected wavelengths, 10.85 μ (estrone), 11.00 μ (equilin), and 10.45 μ (equilenin). Analysis of a number of mixtures of known composition gave very good recoveries for each ketosteroid.

Analysis of the diol fraction presented greater difficulty. This fraction, after the Girard separation, is usually contaminated with nonestrogenic compounds. In addition, estradiol-17β (the only potent diol) is almost always a minor component of this fraction. A second paper from this laboratory (27) reported a procedure for the diols based on partition chromatography and infrared spectrophotometry. The diols were separated on a column of siliceous earth and sodium hydroxide as the stationary phase and benzene as the mobile solvent. Three fractions were obtained containing, respectively, the estradiols, the dihydroequilins, and the dihydroequilenins. Each fraction was converted to the benzenesulfonyl esters and the epimeric pairs were determined by infrared analysis for two component systems, using carbon disulfide solutions.

Pharmaceuticals containing estradiol-17β as the chief active constituent were analyzed using a simplified modification of the above procedures. The Girard separation and chromatographic steps were eliminated. After isolation of the hormones the residue was esterified and made to volume in carbon disulfide. The spectra of the sample solution and a similarly prepared standard were recorded from 9 to 11 μ. Figure 4 shows the difference in spectra of the estradiol



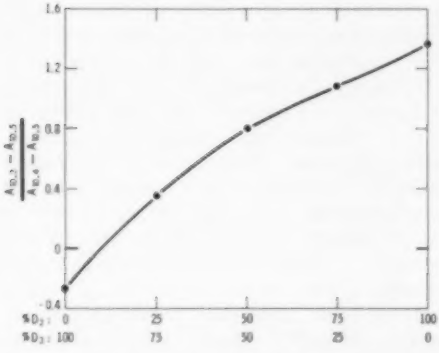


Fig. 4.—The effect of variation of the ratio of vitamin D_2 and D_3 on the infrared absorbance at 10.3–10.4 μ.

isomers. Estimation of estradiol-17β was made by baseline absorbance measurement of the characteristic maximum at 10.10 μ. Accurate results were obtained for estradiol-17β in the presence of significant amounts of the other estrogens.

Analysis of a number of natural products was made by the methods for ketosteroids and diols and the total potencies of the products were calculated using the potencies of the individual estrogens shown in Chart I. The potencies agreed closely with those obtained by biological assay of the same preparations (28).

Mulls.—Sample-handling in the solid state was greatly simplified by the introduction of the "mull." In this technique, a few milligrams of the sample is mulled with a small amount of liquid petrolatum (Nujol) and the resultant paste is pressed between two halide windows. Interference from the mineral oil is significant only in the C—H stretching and CH_2 and CH_3 bending regions. Hexachlorobutadiene and fluorokerosene have also been used for the preparation of mulls. These materials are relatively transparent in regions where liquid petrolatum absorbs, although they absorb strongly at other wavelengths. This procedure is widely used for qualitative work and is also adaptable to quantitative infrared spectrophotometry.

The analysis of crystalline penicillins by Barnes, *et al.* (12), was an important and comprehensive work that did much to emphasize the scope of analytical infrared spectrophotometry. These workers employed the mull technique for the identification of the sodium penicillins G, F, K, X, and amyl, and for the estimation of sodium penicillins G, F, and X.

The quantitative spectra of the five species showed distinct differences and the following bands were chosen as suitable for qualitative absorbance measurements: sodium penicillin G, 14.23 μ; sodium penicillin F, 10.30 μ; sodium penicillin X, 12.03 μ, 8.20 μ; sodium penicillin K, 7.52 μ; sodium penicillin amyl, 14.0 μ, 8.57 μ.

All quantitative spectrophotometric measurements depend upon a relationship that is expressed by the Beer-Lambert law

$$I = I_o e^{-kcd}$$

where I_o and I are the radiant energy falling upon and passing through the sample, k is an absorption coefficient characteristic of the sample, c is a concentration, and d the length of the path through the sample. The authors believed that the determination of thickness in a mull was impractical, and resorted to the use of *dl*-alanine as an internal standard. This material has a strong band at 12.42μ (where the penicillin

salts exhibit weak absorbance) and does not interfere at the selected measuring wavelengths.

Analytical directions were essentially the same for each type of penicillin salt. Weighed amounts of sample or standard (15 mg.) and *dl*-alanine (7.5 mg.) were ground together thoroughly in a small mortar. A portion of the mixture and a drop of liquid petrolatum were placed between two salt windows and mulled until a uniform film was obtained. The film thickness was adjusted by pressure to produce about 0.300 absorbance at the selected measuring wavelength. The absorbance was recorded over a spectral range sufficiently broad to include both penicillin salt and *dl*-alanine measuring maxima. A ratio, R = baseline absorbance of the pencillin salt/-baseline absorbance of the internal standard, was determined for each mull. The concentration of penicillin salt in sample was calculated from the formula

$$\text{Concentration of penicillin salt in sample} = \frac{R \text{ sample}}{R \text{ standard}} \times \text{weight penicillin salt in standard}$$

Results of analysis of known mixtures containing amounts of sodium penicillin G samples showed good accuracy and precision. Similar results were also reported for sodium F and X salts.

The influence of crystal orientation upon absorptivity was noted and investigated by the authors. Their findings are now well known to all users of solid phase sample-handling techniques; deviation from random orientation results in lowering of absorbance value.

Garlock and Grove (29), in their procedure for sodium penicillin G, modified the mull procedure to eliminate the use of an internal standard. A brass spacer (approximately 0.003 inch) was used to control the thickness of the mull. A knowledge of the exact thickness was unnecessary because the same spacer was used for both sample and standard. Weighed amounts of liquid petrolatum and penicillin salt were thoroughly ground together. Sufficient mull was placed between the salt plates so that when pressure was applied to the cell assembly the mixture almost filled the opening in the spacer, but did not touch it. The baseline absorbances of sample and standard were determined as described by Barnes, *et al.* (12).

Results obtained using this method compared favorably with those obtained by chemical and biological methods. Others species (except sodium penicillin X) did not interfere in the assay of sodium penicillin G.

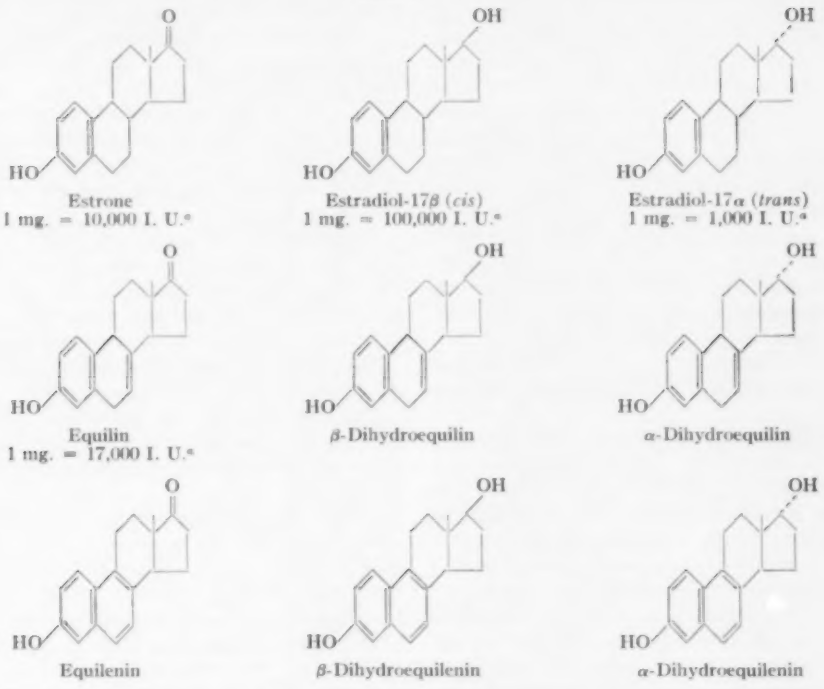
Dispersions in Carbon Disulfide.—In 1951, Dolinsky (30) succeeded in dispersing insoluble substances in carbon disulfide and determined their absorbance in liquid-tight cells of 0.5 to 1.0 mm. thickness. This procedure had all the desirable characteristics of conventional solution sample-handling techniques. Sample solutions were prepared by vigorously shaking 250 mg. of sample, 250 mg. of aluminum stearate, and 25 ml. of carbon disulfide with 50 ml. of small glass beads. The time required to obtain a suitable suspension (particle size 3μ or less) depended upon the sample, but usually was twenty to sixty minutes. The blank solution prepared by dissolving 250 mg. of aluminum stearate in carbon disulfide, required no shaking. The suspensions were sufficiently fluid to be injected into the cells with a hypodermic syringe. The spectra of a number of substances, including sucrose, sulfadiazine, cortisone, and salicylic acid were given. Analysis of two component mixtures of sulfamethazine-sulfamerazine and sucrose-maltose gave satisfactory recoveries. At the time of publication the usefulness of this method was limited because of the large sample required, and the advent of the potassium bromide disk technique discouraged further study. Recently a micro sample procedure requiring only 10 mg. of sample was devised (31) employing a mechanical vibrator shaker

Dispersions in Halides.—The potassium bromide disk method of Schiedt (32) and Stimson (33) has been widely accepted as the procedure of choice for infrared analysis in the solid phase. Many papers have been published reporting the application of this technique to both qualitative and quantitative analysis (8). Acceptable spectra can be made with 1 mg. or less of sample and the transparency of potassium bromide eliminates background interference. As with other procedures for obtaining the spectra of solids, crystal size, form, and orientation must be controlled to avoid anomalous results. Precautions must be taken to avoid anomalies due to reactions between sample and halide.

The Chemical Laboratory of the American Medical Association incorporated the infrared absorption spectrum as a test for identity in their monographs in "New and Nonofficial Drugs." These spectra were made from potassium bromide disks. The monographs, published in *Drug Standards*, contained actual reproductions of each spectrum from 2 to 15 μ. Over forty spectra have been presented.

The U. S. P. for the first time included infrared tests for identity in its sixteenth revision. Eighty-nine pharmaceuticals were involved and the majority employed the potassium bromide

CHART I



[a] The potency of estrone was set by international agreement as 1 mg. = 10,000 I. U. Other potencies are unofficial. Those given in chart were determined by using rats as test animals and estrone as standard (F. D. A. Division of Pharmacology). Equilenin and the dihydro derivatives of equilin and equilenin are essentially inactive.

disk technique. Recognizing that a spectrum made under varying conditions and instrumentation might not match a published spectrum, the U. S. P. directed as follows: "The infrared absorption spectrum of a potassium bromide dispersion of ——— exhibits absorpt on maxima on y at the same wavelengths as that of a similar preparation of U. S. P. ——— Reference Standard."

Rosenkrantz, *et al.* (34), utilized the disk technique to determine a number of steroid hormones including several binary mixtures. Sample and halide were mixed with a spatula prior to pressing, and recoveries within 8 per cent of the known amounts were reported.

Quantitative disk procedures have been reported by Hayden and Sammul (35) for cortisone, hydrocortisone, ethisterone, caffeine, acetophenetidin, warfarin, and meprobamate. The authors stressed the necessity of following a definite routine to obtain reproducible results. An aliquot of the sample in a volatile solvent was hand ground with potassium bromide until the solvent evaporated (five to ten minutes). Alternatively, residues of a iquots were ground with the halide in a vibrator grinder for an accurately measured time. The mixtures were dried, pressed into disks, heated at 105° for fifteen to thirty minutes, cooled to room temperature, and their absorption spectra recorded from 2 to 15 μ. Sample and standard were prepared in an identical manner and the quantitative calculations were made from a modified Beer-Lambert law equation

$$K = (A \times H)/(C \times L)$$

where K = absorption coefficient for the substance, A = baseline absorbance at selected band, H = band width at one-half the height of absorbance, C = concentration, and L = thickness of disk area exposed to beam.

The results of analysis of a number of commercial pharmaceutical preparations compared very favorably with those obtained by other conventional assays. Results of analysis of known mixtures agreed within plus or minus 3 per cent.

This laboratory (36) has effected the rapid

separation and identification of microgram quantities of compounds by combining paper chromatography and infrared spectrophotometry. Quantities of pharmaceuticals in excess of 500 mcg. were extracted from the paper and determined directly by the normal disk procedure. Smaller quantities were ground with 40 mg. of potassium bromide and pressed into 3 × 10 mm. windows in paper, as suggested by Pickering (37) and Muller (38). Potassium bromide peripheries were also used in place of the paper. A blank was prepared by extracting from the chromatogram a paper zone equivalent to the sample zone. A disk made of this blank was used in the reference beam to compensate for impurities. Recoveries better than plus or minus 10 per cent were obtained for cortisone-hydrocortisone mixtures.

Bonnichsen and co-workers (39) reported a micro procedure for caffeine, antipyrine, and acetophenetidin. They employed a novel use of the potassium bromide disk. The material to be identified was sublimed onto a blank disk and the infrared spectra were recorded using a reflecting microscopic accessory.

The appearance of an occasional anomalous spectrum has caused concern for users of the halide disk technique. Baker (40) attributed these anomalies to (*a*) formation of polymorphic crystalline forms, (*b*) formation of amorphous materials, (*c*) formation of hydrates, and (*d*) interaction of sample and halide. Prolonged grinding, either mechanical or by hand, appeared to accelerate these changes. Roberts (41) working with steroid hormones concluded that most uniform results were obtained with mechanical vibrator grinding. Hayden and Sammul (42) made an extensive study of the use of the potassium bromide disk method for the quantitative assay of cinchona alkaloids. They found that the spectra of quinine and quinidine were readily modified by drastic conditions during disk formation. Cinchonine and cinchonidine, however, produced uniform spectra under a variety of conditions. Jensen (43) studied the effects of vibrator grinding and repressing on 16 pharmaceuticals of widely different structures. Grinding periods of five minutes produced maximum baseline absorbance (at the selected measuring band) for most compounds. Pressing followed by grinding in an agate mortar and repressing increased the baseline absorbance of most compounds. Two to five repetitions were necessary to achieve maximum absorbance. Phenobarbital disks prepared by vibrator grinding were not reproducible. Work in this laboratory corroborates this finding.

A method has been found in this laboratory (44) to maintain the desired polymorphic form of a substance in the halide disk. Starting with a single form and using a very short grinding time, the presence of a specific solvent prevented transition. In the case of quinidine, heptane or benzene were the best stabilizing solvents. These solvents have been satisfactory for other polymorphic substances but they may not be universally applicable.

The interaction of sample and halide is also being studied (45). When disks prepared from alkaloid sulfates were heated to 105° prior to scanning, the compound was converted to the halide salt. This change occurred with quinine sulfate when the halide was sodium chloride, potassium bromide, or potassium iodide. Unheated disks showed no interaction. Other workers (46) have observed this interaction between hydrochloride samples and the potassium bromide disk.

Gases.—Infrared spectrophotometry is an excellent tool for the analysis of gases since sample-handling is no problem and spectra are sharp and detailed. Extreme variations in concentration can be accommodated since cells having effective lengths as great as 50 meters are available. Few gases are classed as pharmaceuticals. Of these, nitrous oxide, cyclopropane, carbon dioxide, helium, oxygen, ethylene, and ethyl chloride are best known. Oxygen, being a diatomic molecule, has no infrared spectrum. The low boiling liquid anesthetics such as ethyl ether, vinyl ether, chloroform, and 2-bromo-2-chloro-1,1,1,-trifluoroethane, can be handled as gases under reduced pressure. An excellent article by Pierson, *et al.* (47), presents the qualitative spectra of 66 gases, including most of the above mentioned. Quantitative infrared procedures for medicinal gases apparently have not received extensive study.

Identification of Unknown Pharmaceuticals.—An important use of infrared spectrophotometry, rarely reported in the literature, involves the identification of unknown materials. Federal, state, and municipal regulatory agency laboratories are frequently called upon to analyze samples in this category. Quite often the sample is a dosage form of a pharmaceutical and consists of a single tablet or capsule. Preliminary separations and purifications are almost always necessary before spectrophotometric examinations can be made. The isolation of the active component(s) is most commonly made by partitioning between immiscible solvents, or by chromatography. In some cases both procedures are required. If more than one com-

pound is indicated or suspected, they should be separated if possible

The infrared spectrum of the separated substance(s) will yield much information regarding its chemical composition. The interpretation will, however, require a knowledge of the relationship of spectra to structures and a library of infrared spectra of pharmaceuticals. The presence or absence of such functional groups as hydroxyl, amine, and carbonyl (ketones, aldehydes, anhydrides, etc.) is readily apparent. The possibility of unsaturation and benzenoid structure is revealed by a study of the C—H stretching region and the absorption between 6 and 7 μ. The position and number of substitutions in the benzene ring are indicated in the 5 to 6 μ spectrum. Ethers, esters, and carboxylic acids are not difficult to recognize. Many other structural features may be indicated but not positively identified. When all the facts concerning the spectrum of the unknown are assembled, a "guess" as to its identity or type is made. Identity is established by matching the spectrum of the unknown with that of an authentic substance. The spectra of the known and unknown should be made with identical instrumentation and sample-handling.

CONCLUSIONS

The applications discussed in this review represent but a few of the uses of infrared spectrophotometry in the pharmaceutical laboratory. In the large drug manufacturing establishments, the infrared spectrophotometer is used in the development and synthesis of new pharmaceuticals, and in many operations in the plant for process control as well as quality control. Many universities giving courses in pharmaceutical chemistry use infrared spectrophotometry as a teaching adjunct. Commercial analysts, as well as governmental regulatory agencies, use infrared spectra as legal evidence in court cases involving pharmaceuticals.

As techniques in methods and instrumentation improve, the use of infrared spectrophotometry in the pharmaceutical field will increase. The U. S. P. XVI contains numerous qualitative infrared tests for identity but only two quantitative procedures. The next revision of this compendium will no doubt have many more infrared assays. Recent advances in vapor-phase chromatography have resulted in separations of high melting alkaloids and hormones (48). This mode of separation, combined with infrared spectrophotometric estimation, should result in many versatile and specific procedures.

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Optical Crystallographic Properties of Some Glucocorticoids

By JOHN A. BILES

The optical crystallographic properties of 17 water-insoluble glucocorticoids are reported. These data are of value for purposes of identification, determination of purity, and the data could be used in helping to determine molecular orientation in X-ray analysis as evidenced by the work of Bernal (1). The molar refraction values for the 17 compounds have been experimentally determined and compared with the calculated values. Attention has been called to the variation of the atomic refraction value for fluorine. Photomicrographs of characteristic crystals of the 17 steroids are included.

THE USE OF crystallographic techniques continues to attract attention from systematic workers (2–8). Optical crystallographic properties, X-ray crystallography, and fusion analysis have been used for the study of steroids. Winchell (9) has tabulated the optical properties of some steroids. Shell (10) has reported the optical and X-ray properties of hydrocortisone acetate. The unit cell dimensions and X-ray powder data for prednisolone acetate have been reported by Pasternak (11). In a series of papers Parsons and co-workers recorded the X-ray diffraction powder data for 138 steroids (12–15). Gilpin, applying the McCrone technique of fusion analysis, observed that fusion analysis was an excellent means for the identification of some sterols and could be done by observers ignorant of optical crystallographic techniques (16).

In 1932 Bernal (1) found "essential similarities of properties" of ergosterol and its irradiation products. He reported that the rings lie approximately in the *bc* plane, that of the larger refractive indexes. The five sterols studied were monoclinic, optically positive, and showed a distinct tendency to elongation along the *b* axis. The unit cells were closely related. From the X-ray and optical data, Bernal concluded that the earlier suggested formulas used to denote the structure of vitamin D type compounds were not correct. This work played an important role in the elucidation of the correct structure. Following this report, the X-ray crystallographic and some optical properties for 80 sterols were reported (17).

With the exception of the reported data for hydrocortisone acetate (10) and the reported data for the polymorphic forms of cortisone acetate (32), no reports have been made concerning the optical crystallographic properties of the glucocorticoids. The present communication reports the optical crystallographic data of some commonly used steroids, presents photomicrographs of these steroids, and reports the density and molar refraction values. The data for the ter-butyl-acetates of hydrocortisone and prednisolone will be the subject of a separate communication.

EXPERIMENTAL

A detailed procedure for the determination of the optical crystallographic properties, the techniques, equipment, and immersion oils have been previously described (18). The photomicrographs illustrated in Fig. 1 were obtained by the procedure previously described (19). Crystals of the steroids were obtained by dissolving the steroid in a small quantity of hot ethanol. After solution of the steroid occurred, enough hot distilled water was added to make the final solvent approximately 50% ethanol. Excellent crystals were obtained as demonstrated by the photomicrographs. It must be emphasized that with variation of solvent, different phases will likely occur. Polymorphism of steroids is well known among those working with these compounds (20, 21). The optical crystallographic data for the water insoluble glucocorticoids are recorded in Table I. The axial angle, $2V$, was calculated using the formulas suggested by Wahlstrom (22).

The density of the steroids was determined using the flotation technique. Thoulet's solution was prepared and used in determining the density (23). The density of the liquids was determined using a 10-ml. Gay-Lussac specific gravity bottle. The

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TABLE I.—THE OPTICAL CRYSTALLOGRAPHIC PROPERTIES OF SOME STEROIDS

Compound	System	Crystal Habit	Optic Sign	Axial[a] Angle	Optic[b] Orientation	Dispersion	Refractive Indexes α (ω)	β (ϵ)	γ
Cortisone alcohol	Orthorhombic[c]	Lamellar	+	62°	$XX \parallel b$ $YY \parallel c$ $ZZ \parallel a$	$r > v$[d]	1.560	1.578	1.631
Hydrocortisone alcohol	Orthorhombic	Tabular	+	59°	$XX \parallel b$ $YY \parallel a$ $ZZ \parallel c$	$r > v$	1.535	1.562	1.650
Hydrocortisone cyclopentylpropionate	Hexagonal	Columnar	+	0°	$\omega \parallel a$ $\epsilon \parallel c$	None observed	1.551	1.564	...
Fludrocortisone alcohol	Orthorhombic	Columnar	+	52°	$XX \parallel c$ $YY \parallel a$ $ZZ \parallel b$	$r > v$ Strong	1.575	1.588	1.646
Fludrocortisone acetate	Tetragonal	Tabular	−	0°	$\omega \parallel a$ $\epsilon \parallel c$	None observed	1.604	1.538	...
Prednisolone alcohol	Orthorhombic	Tabular	+	48°	$XX \parallel b$ $YY \parallel c$ $ZZ \parallel a$	$v > r$	1.573	1.586	1.654
Prednisolone acetate	Monoclinic	Prismatic	+	88°	$YY \parallel b$ $ZZ \wedge c$ 35°	$v > r$	1.522	1.583	1.657
Prednisone alcohol (*A*)	Orthorhombic	Tabular	+	21°	$XX \parallel b$ $YY \parallel c$ $ZZ \parallel a$	$r > v$ Strong	1.582	1.584	1.649
Prednisone alcohol (*B*)	Orthorhombic	Columnar	+	61°	$XX \parallel a$ $YY \parallel c$ $ZZ \parallel b$	$r > v$ Slight	1.486	1.516	1.615
Prednisone acetate	Monoclinic	Tabular	+	35°	$XX \wedge c$ 29° $YY \parallel b$	$v > r$	1.566	1.572	1.637
Methylprednisolone alcohol	Monoclinic	Tabular	−	76°	$YY \parallel b$ $ZZ \wedge c$ 13°	$r > v$	1.532	1.602	1.649
Methylprednisolone acetate	Orthorhombic	Tabular elongated	+	38°	$XX \parallel b$ $YY \parallel a$ $ZZ \parallel c$	$v > r$	1.562	1.575	1.700
Fluorometholone	Monoclinic	Columnar	+	26°	$XX \parallel b$ $YY \wedge c$ 12°	$r > v$ Strong horizontal	1.562	1.568	1.704
Dexamethasone	Orthorhombic[c]	Tabular	+	50°	$XX \parallel a$[e] $YY \parallel c$ $ZZ \parallel b$	$r > v$ Strong	1.559	1.574	1.649
Triamcinolone	Orthorhombic	Columnar	+	52°	$XX \parallel a$[f] $YY \parallel c$ $ZZ \parallel b$	None observed	1.561	1.582	1.680
Triamcinolone acetonide	Trigonal	Columnar	−	0°	$\omega \parallel a$ $\epsilon \parallel c$	None observed	1.595	1.546	...
Triamcinolone diacetate	Orthorhombic	Columnar	−	69°	$XX \parallel c$ $YY \parallel a$ $ZZ \parallel b$	None observed	1.517	1.567	1.592
Dichlorisone acetate	Monoclinic	Tabular	+	59°	$XX \wedge c$ 37° $\gamma\gamma \parallel b$	None observed	1.561	1.586	1.698

[a] 2V calculated. [b] Assigned according to crystal habit. [c] Symmetric extinction. [d] Dispersion of optic axes of orthorhombic crystals is axial. [e] $c < a < b$. [f] Optic normal shows symmetric extinction.

weight of the volume of water was determined at the same time the weight of the equal volume of adjusted liquid was determined.

The experimental molar refractions were determined using the Lorenz and Lorentz equation (24). The refractive index value used in calculating the molar refraction, Mr_D, was determined from $\sqrt[3]{\alpha\beta\gamma}$ for the biaxial crystals and from $\sqrt[3]{\omega^2\epsilon}$ for the uniaxial crystals. It should be emphasized that the refractive indexes were determined using monochromatic light (18). The calculated molar refractions were obtained using the commonly accepted values for atomic refractions (24). The densities and molar refractions are recorded in Table II.

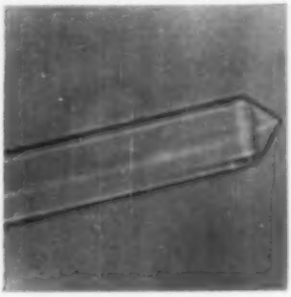
DISCUSSION

Examination of the optical crystallographic properties of the steroids in Table I indicates that these data are of value for purposes of identification. Crystallographers often assume that because most molecules of organic compounds are complex, the symmetry of the crystal would be of low order. Thus, it would be expected that such compounds would crystallize in the monoclinic system. It is therefore surprising to some crystallographers that some of the steroids studied crystallize in systems of high symmetry as exemplified by the tetragonal and hexagonal phases of fludrocortisone acetate, hydrocortisone cyclopentylpropionate, and triamcinolone acetonide. Most of the steroids in Table I are orthorhombic and most of the steroids studied are positive crystals.

Prednisone alcohol was found to be dimorphic. The different phases were not isolated. Thus, the customary method to identify the phases was not applied since it could not be determined which phase was more stable. It was also noted that some of the crystals of prednisone acetate showed dispersion $r > v$. By far the greater number of crystals

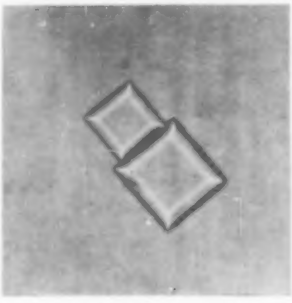
CORTISONE ALCOHOL

HYDROCORTISONE ALCOHOL

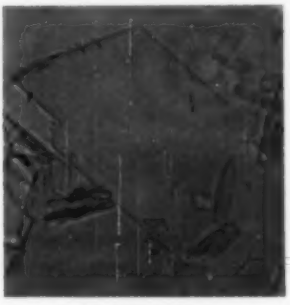


HYDROCORTISONE CYCLOPENTYLPROPIONATE

FLUDROCORTISONE ALCOHOL



FLUDROCORTISONE ACETATE



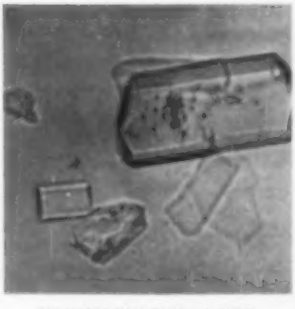
PREDNISOLONE ALCOHOL

PREDNISOLONE ACETATE

PREDNISONE ALCOHOL (A)

PREDNISONE ACETATE

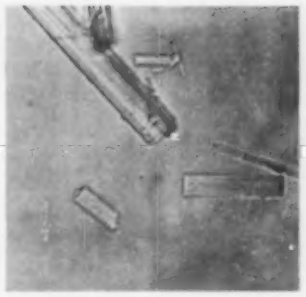
FLUOROMETHOLONE



METHYLPREDNISOLONE ALCOHOL

METHYLPREDNISOLONE ACETATE

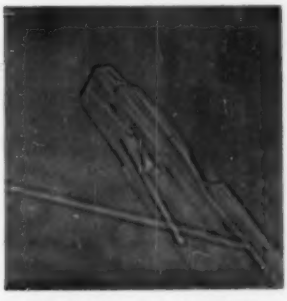
TRIAMCINOLONE



TRIAMCINOLONE DIACETATE

TRIAMCINOLONE ACETONIDE

DEXAMETHASONE



DICHLORISONE ACETATE

Fig. 1.—Photomicrographs showing the crystal habits of some steroids. 350×

TABLE II.—DENSITIES AND MOLAR REFRACTIONS FOR SOME STEROIDS

Compound	Density	$\sqrt[3]{\alpha\beta\gamma}$ or $\sqrt[3]{\omega^2\epsilon}$	Experimental MrD	Calculated MrD
Cortisone alcohol	1.327	1.526	91.53	92.99
Hydrocortisone alcohol	1.261	1.582	95.87	94.51
Hydrocortisone cyclopentylpropionate	1.208	1.555	129.35	129.26
Fludrocortisone alcohol	1.380	1.603	94.67	94.44
Fludrocortisone acetate	1.343	1.582	105.00	103.39
Prednisolone alcohol	1.337	1.604	92.73	94.04
Prednisolone acetate	1.279	1.583	105.1	103.4
Prednisone alcohol (*A*)	1.334	1.605	92.48	92.53
Prednisone acetate	1.293	1.591	102.98	102.77
Methylprednisolone alcohol	1.262	1.594	100.63	98.66
Methylprednisolone acetate	1.328	1.611	108.87	108.02
Fluorometholone	1.322	1.610	98.99	98.16
Dexamethasone	1.337	1.594	99.64	98.59
Triamcinolone	1.426	1.607	95.50	95.50
Triamcinolone acetonide	1.323	1.578	109.08	107.39
Triamcinolone diacetate	1.379	1.558	111.90	113.99
Dichlorisone acetate	1.423	1.614	111.52	111.50

showed dispersion $v > r$. The crystals showing the two types of dispersion could not be separated by microscopic examination. It is possible that two phases of prednisone acetate exist with very similar optical crystallographic properties other than dispersion. However, it should be observed that prednisone alcohol might be present in a very small amount. Both the alcohol and the acetate are tabular as is demonstrated by the photomicrographs.

The birefringence of the crystal is obtained by taking the difference between the highest and lowest refractive index. The limits were found to be 0.013 (hydrocortisone cyclopentylpropionate) and 0.142 (fluorometholone). A great many of the steroids showed birefringence of the order of 0.07 or 0.13. There was no correlation of the birefringence between the free alchol and the ester form.

Hamlin and co-workers have recently reported the conversion of prednisolone to hydrocortisone (25). Examination of the optical properties of these two steroids shows that the crystallographer can easily identify one steroid in the presence of the other. Triamcinolone can be easily distinguished from dexamethasone. The free alcohols can be easily distinguished from the esters. Thus, these data are very valuable for determination of purity. The optical crystallographic properties may also be of value in determining molecular orientations in the crystalline state in the manner that Bernal used such properties for determining the orientation of vitamin D compounds in the crystalline state and elucidating the structure of the steroid.

In determining the experimental molar refraction values of the fluorinated steroids, it was difficult to

arrive at a valid conclusion for the atomic refraction value for fluorine. Park stated that the value varied from 0.8 to 1.4 (26). For some fluorinated hydrocarbons, Henne and Zimmerschied reported atomic refraction values between 0.90 and 1.44 (27). Fainberg used an atomic refraction value of 0.92 for fluorine (28). Nodiff, *et al.*, stated that the atomic refraction of fluorine was 1.23 (29). Park and associates used the value of 1.03 for fluorine (30). From the reports in the literature it is evident that fluorine presents a special case. For the purposes of calculating the molar refraction values, the atomic refraction value of 1.03 was arbitrarily selected.

An examination of Table II indicates that there is a close relationship between the calculated and experimental Mr_D values. The average deviation was found to be 0.83%. Triamcinolone diacetate showed the maximum deviation of 1.76%. It has been reported that triamcinolone diacetate crystals are solvated and as such melt at 186–188° with effervescence. After drying, the crystals melt at 235° (31). The triamcinolone diacetate used in determining the optical properties was found to melt at 168° (Kofler block). The material was dried in an Abderhalden apparatus under vacuum at 137° for four hours. For a sample weighing 0.216 Gm., the loss in weight was 9 mg. There was no change in the crystallographic properties after drying.

CONCLUSIONS

1. The optical crystallographic properties of 17 water-insoluble glucocorticoids are reported.

2. Photomicrographs of typical crystals are shown in Fig. 1.

3. The densities have been reported for the 17 steroids studied.

4. The molar refractions have been experimentally determined and calculated. The status of the atomic refraction value for fluorine has been briefly summarized.

5. The reported data should be of definite value in (*a*) identification, (*b*) determination of purity, and (*c*) X-ray analysis of steroids and determination of molecular orientation.

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Single-Injection Method for the Assay of Digitalis in Pigeons

By HERBERT A. BRAUN, LEHMAN M. LUSKY, and STANLEY E. SRENSEK

A method for the biological assay of digitalis in pigeons is described. It is based on the comparison of the LD_{50} obtained at three dosage levels for the U. S. P. Digitalis Reference Standard (1942) and for the digitalis preparation being assayed. Results of assays with 15 digitalis preparations are in good agreement with those obtained by the U. S. P. XV pigeon method and by the U. S. P. XIII cat method. The procedure for the method is simple but is more time-consuming than the present method.

MOST OF THE biological methods which have been employed for assaying digitalis and related drugs depend on the determination of the fatal dose. This is true of the present U. S. P. intravenous pigeon method, which over the past decade has been found to be satisfactory because it has yielded comparable and reproduceable results in the various industrial and governmental laboratories where it has been utilized.

Hatcher (1) in 1912 first reported results on the mean fatal dose for a number of digitalis tinctures assayed by a single-injection method in cats. Hanzlik (2) in 1929, while working on the pigeon emetic method, demonstrated that for several tinctures of digitalis there was remarkable agreement between the minimum fatal dose for the cat and for the pigeon. Haag and Woodley (3) compared the minimum lethal doses of their intravenous pigeon method with results obtained on the cat by the Hatcher-Brody method. They found the minimum lethal dose by the pigeon infusion method to be 25 per cent higher than by the cat method.

The procedure for the standardization of digitalis has changed greatly since the publication of these papers. Improvements such as the use of a standard of comparison, the injection of the test solution on the basis of the weight of the animal, the specification of the rate of injection, and the application of statistical analysis to the results are but a few of the factors which have contributed to the advancement of modern bioassay procedure.

Our interest in a single-injection method was twofold. First, that it might be used successfully for the standardization of digitalis; and second, to determine whether such a method would yield results comparable to those obtained with the U. S. P. XIII cat method and with the present U. S. P. repeated intravenous infusion pigeon method.

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METHOD

The procedure which was utilized by us in this single-injection method was based on the comparison of the LD_{50}'s obtained from three dosage levels for the U. S. P. Digitalis Reference Standard (1942) and for the digitalis preparation being assayed. The three-dosage method is commonly employed in the determination of the LD_{50} of compounds in bioassay practice.

Adult common pigeons weighing between 280 and 410 Gm. were selected at random for these experiments. Birds which appeared malnutritioned or emaciated were discarded. They were fed *ad libitum* on a diet of commercial pigeon feed, grit, and water for a week prior to use. The birds were fasted for eighteen to twenty-four hours prior to the test.

In the performance of the assay, each of the three doses of the tincture diluted with 0.9% sodium chloride was made up separately. All doses used were spaced at equal logarithmic intervals. The doses selected were diluted so that the volume of diluted tincture injected was equivalent to 2.0 ml. per 100 Gm. of body weight. Thus the amount injected into a 300-Gm. pigeon was 6.0 ml. of the dilution. In our experience, 5.0 ml. of tincture per 100 ml. of dilution sufficed as a first approximation of the median dose for the standard.

The doses of the tincture were infused directly into the alar vein of the pigeon under pressure from a 10-ml. semimicroburet calibrated in 0.05 ml. Injections of the diluted tincture were completed within one minute.

At least six pigeons per dose or a total of 18 pigeons were utilized for the completed test of a preparation of the standard or of the unknown. The pigeons were observed for two hours after the administration of the diluted tincture. Only those birds which died within this period were included in the determination of an LD_{50} of a preparation. Actually, with few exceptions, death of the pigeons occurred in from four to sixty-seven minutes. Calculation of the LD_{50} was made by the method of least squares.

Assays on 15 digitalis preparations have been completed by the single-injection method, the present U. S. P. pigeon method, and the U. S. P. XIII cat method. The digitalis preparations represented various types of preparations on the market. Whenever necessary a tincture was prepared so that a single batch could be used for all three assays. Assays by all three methods were completed within fourteen days.

TABLE I.—COMPARISON OF THE POTENCY ESTIMATES OF VARIOUS TYPES OF DIGITALIS PREPARATIONS BY THREE METHODS OF ASSAY

Type of Preparation	Preparation	Percentage Potency ± Standard Error: Single-Injection Method	U. S. P. Intravenous Pigeon Method	U. S. P. XIII Cat Method
Powdered leaves	A	136.3 ± 2.4	128.2 ± 4.2	138.3 ± 5.7
	B	131.6 ± 2.4	138.1 ± 4.6	145.4 ± 5.1
	C	113.1 ± 2.2	102.2 ± 5.8	92.0 ± 6.1
	D	73.8 ± 1.6	67.4 ± 5.0	76.1 ± 7.3
	E	111.1 ± 1.8	116.7 ± 4.1	104.6 ± 5.9
	F	87.8 ± 2.0	85.1 ± 8.0	84.9 ± 6.6
Tablets	A	72.5 ± 1.8	70.4 ± 7.0	67.8 ± 7.5
	B	58.6 ± 1.0	65.1 ± 4.2	62.2 ± 7.2
	C	96.3 ± 2.9	101.0 ± 5.2	103.2 ± 7.3
	D	98.4 ± 3.3	98.1 ± 4.0	100.7 ± 7.0
	E	91.7 ± 2.1	97.2 ± 4.7	105.7 ± 7.4
Pills		92.9 ± 2.0	101.0 ± 4.0	103.2 ± 7.1
Tincture		118.8 ± 1.8	112.0 ± 4.3	120.0 ± 7.3
Purified glycosides[a]	A	107.6 ± 3.8	94.6 ± 6.4	106.6 ± 7.0
	B	94.3 ± 1.9	108.4 ± 4.3	106.7 ± 7.0

[a] Purified extracts of cardio-active glycosides of digitalis leaf.

All preparations assayed were commercial samples except powdered leaves A and F. Powdered leaf A is the International Standard (1936) while F is the New York Heart Association Powder No. 7. The New York Heart Association Powder No. 7 is an atypical powder yielding a potency of 62% by the intravenous guinea pig method and 57% by the frog method, but it shows good agreement by the three methods reported in this paper.

RESULTS

A comparison of the results obtained by assaying the various types of digitalis preparations is shown in Table I.

The data in this series of assays reveal that the potencies obtained by one method are not consistently higher or lower than the results obtained by either of the other methods, in fact, there is satisfactory agreement between the results of all three methods.

The percentage difference between the potencies of the 15 digitalis preparations by the present U. S. P. intravenous pigeon method and the single injection method indicated that in no instance did they differ by more than 14%.

Statistical comparison of the results obtained on the 15 samples assayed by the present U. S. P. infusion method and by the single-injection method showed that there is no significant difference between the potencies so obtained.

The standard errors of assay by the single injection method are approximately one-half those obtained when the U. S. P. intravenous infusion method was used. This is a consequence of the significantly greater slopes obtained and the greater number of animals used in this method.

Table II shows the LD_{50}'s and the slopes observed for 10 consecutive assays of the U. S. P. Digitalis Reference Standard (1942) completed during the fall, winter, and spring seasons by the single-dose method. The LD_{50} of the U. S. P. Digitalis Reference Standard (1942) varied from 102.3 mg./Kg. to 110.8 mg./Kg., a difference of approximately 8.3%.

A comparable series of 12 assays of the U. S. P. Digitalis Reference Standard (1942) completed by the present U. S. P. pigeon assay during the same seasons yielded LD_{50}'s varying from 86.7 mg./Kg. to 104.0 mg./Kg., a difference of approximately 20%. The average LD_{50} for the U. S. P. Digitalis Reference Standard (1942) by the present U. S. P. pigeon method was 96.2 mg./Kg. as contrasted with 104.7 mg./Kg. for the single injection method, a difference of 8.8%.

TABLE II.—LD_{50}'S AND SLOPES OBTAINED WITH THE U. S. P. DIGITALIS REFERENCE STANDARD WITHIN AN EIGHT-MONTH PERIOD

Test	LD_{50} in mg./Kg.	Slope (Probits/Log Dose)
1	108.14	35.22
2	103.62	65.18
3	110.84	31.94
4	102.12	71.90
5	102.31	28.51
6	103.54	14.72
7	103.88	64.32
8	104.76	28.63
9	104.00	53.69
10	103.52	64.12
		Mean slope ± S. E. = 45.823 ± 6.378

The data on the slope in Table II, as determined by the method of least squares, were included to demonstrate the sensitivity of pigeons to increments of dosage of digitalis when this drug was administered as a single injection. Whereas the mean slope for the 12 determinations of the U. S. P. Digitalis Reference Standard (1942) by the infusion method was 22.5 probits/log dose, it was 45.8 probits/log dose when the single injection method was used. Comparison of the mean slopes statistically showed their difference to be significant with a p value of 0.035. As a result of the steepness of the slope, the standard errors of the potencies by the single-injection method are small.

DISCUSSION

Gold and co-workers (4) in 1941 showed that the results obtained with digitalis preparations by the U. S. P. XIII cat method paralleled assays on humans. Braun and Lusky (5) demonstrated that there were no significant differences in the potencies of digitalis preparations assayed by the cat method

and the present U. S. P. pigeon method. They stated that it was to be expected that the results of the present U. S. P. pigeon method would show similar agreement with the human assay. The same conclusion can be reached with the single-injection method where results do not differ from those obtained with the U. S. P. XIII cat method and the present U. S. P. pigeon method.

In the evaluation of a bioassay for general laboratory use, a number of factors must be taken into consideration. One of the more important is simplicity of technique. The procedure involved in the determination of the LD_{50} by the single-injection method is one of the simplest and most commonly used techniques in pharmacological procedure. It is no more difficult than the present procedure used in the U. S. P. digitalis assay. However, the LD_{50} method is at times more time-consuming, especially when preparations weaker or stronger than the standard are being assayed. Since the dosage increments are so small, it is advisable to run a preliminary dosage-response curve, using one pigeon per dose, in order to establish the proper test doses. This is not the case with the present U. S. P. assay, where the proper dose for injection can generally be found by performing a preliminary assay on two pigeons.

The single injection method is more costly than the present U. S. P. pigeon method. Usually, with the present method, six or seven pigeons suffice for the test of each preparation, while with the single-injection method at least 18 pigeons are necessary to establish the LD_{50}. However, since the pigeon is one of the cheapest laboratory animals, the cost of animals used in an assay by the single-injection method, though greater than that of the present, is considerably less than the cost of a cat assay.

SUMMARY

1. The percentage difference between the potencies of 15 digitalis preparations assayed by the present U. S. P. pigeon method and the single-injection method does not differ by more than 14%.

2. The standard errors of assay by the single-injection method are approximately one-half those obtained by the present U. S. P. pigeon infusion method because of the greater slopes and the greater number of animals used in this method of assay.

3. Although the technique employed in the single-injection method is simple, the assay is more time-consuming overall and more costly than the present U. S. P. digitalis assay.

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Triterpenes of *Poria obliqua*

By LEMONT B. KIER

A water extract of the fungus *Poria obliqua* has been shown to possess tumor-inhibiting activity against two test tumors. Two triterpene alcohols were isolated from the hexane extract, one of which was identified as lanosterol. The second, named obliquol, $C_{30}H_{50}O_2$, was shown to have two hydroxyl groups and two double bonds, one of which is inert to hydrogenation. Spectral data indicated that obliquol has a lanosterol-type skeleton. A possible structure for obliquol was considered but eliminated by mixed melting point comparison.

PORIA OBLIQUA (Bres.) is a birch tree fungus found in Poland (1). An American variety is known and a cursory comparison of the two has been made (2). Water extracts of the Polish variety have been used clinically in Poland for the treatment of some malignant tumors (3–6), although the reported results have been inconsistent from the various agencies. It was felt that there must be some merit to these reports and that there should be an accurate evaluation of the extract under carefully controlled testing conditions. Accordingly, the residue from the aqueous extract was submitted from this laboratory to the National Institute of Health for screening. Their preliminary tests indicated that there is a tumor inhibiting action within the acceptable limits for natural products against Sarcoma 180 and Carcinoma 755. The dose required for this action was below the toxic dose in the test animals. The extract had no activity against Leukemia 1210.

Concurrent with this testing, a routine phytochemical study was initiated. From the hexane

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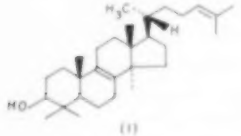
extract were isolated three compounds, of which two could be obtained in sufficient purity for further study. The first compound was thought to be a triterpene based on its molecular formula, $C_{30}H_{50}O_2$, solubility characteristics, color tests, and infrared spectrum. A search of the literature revealed that the compound was new, hence the name obliquol was assigned to it. Perbenzoic acid titration (7, 8) showed that the compound consumed two equivalents of oxygen, indicating the presence of two double bonds, further suggesting that the compound a was tetracyclic triterpene. The infrared spectrum failed to show the presence of this unsaturation but did indicate the presence of two hydroxyl groups, one of which was primary. The tetranitromethane test color, the reaction with only three bromine atoms, and the hydrogenation to only a dihydro compound indicated that one of the double bonds was active, probably trisubstituted, since no unsaturation was indicated in the infrared spectrum, and that the other double bond was inert, in all probability tetrasubstituted.

Additional light was shed upon the structure of obliquol when the remarkable similarity in the infrared spectra was noted between it and a second triterpene alcohol isolated in the same fraction. This second compound was subsequently identified as lanosterol (I). Also, the ultraviolet absorption of obliquol showed low order absorption at 244 and 252 mμ characteristic of the $\Delta^{7,9}$ conjugated diene system (II) which frequently accompanies triterpenes of the lanosterol skeleton as an impurity. Jones and his group (9–12) have used this finding as partial evidence of the presence of a compound with the lanosterol skeleton.

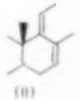
Blandon (13) has used the 205-225 mμ region to characterize the double bond environment of sterols. The absorption curves of obliquol, dihydroobliquol, and lanosterol were plotted and compared to Blandon's curves. All three fell within the range of tetrasubstituted double bonds but the most significant finding was the fact that the curves of lanosterol and obliquol were identical, indicating that the double bond environment of the two are the same.

Further confirmation of this was found using another test for the characterization of the double bond environment. This method, developed by Heilbronner (14), is the measurement of the visible range absorption of the complex formed between the compound and tetranitromethane. By plotting the extinction coefficient against the wavelength, it was observed again that obliquol, dihydroobliquol, and lanosterol had curves in the region assigned to tetrasubstituted double bonds. More important, however, was the finding that the curves of obliquol and lanosterol were identical.

It was now felt, with little doubt, that obliquol possessed the same basic skeleton as lanosterol. Halsall and his co-workers (15) have reported a compound, $\Delta^{8,24}$-lanostadiene-3β,21-ol (III), obtained synthetically by the reduction of methyl pinicolate A, (IV), and having the constants, m. p. 189–192°, $[\alpha]_D + 57°$. Since this synthesized triterpene diol has the lanosterol skeleton, has one primary hydroxyl group, and constants nearly identical to those of obliquol, it appeared likely that obliquol might be $\Delta^{8,24}$-lanostadiene-3β,21-ol.[1] A mixed melting point with obliquol gave a definite depression indicating that obliquol has some structure other than (III).



(I)



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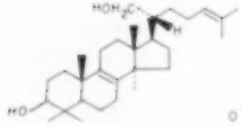
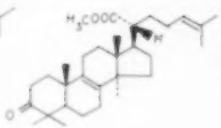


(III)



(IV)

EXPERIMENTAL

All melting points reported were taken on the Kofler apparatus unless otherwise stated. Analyses were performed by the Weiler and Strauss Microanalytical Laboratory, Oxford, England. Spectral work was done by the Department of Chemistry, University of Florida.

Extraction and Fractionation

The dried, powdered, sterile conks (2.4 Kg.) were extracted with hexane for forty-eight hours in a Soxhlet extractor. The extract was concentrated to dryness leaving 12 Gm. of residue (0.5%). This residue was warmed with several portions of dry ether and filtered, leaving a white, granular, insoluble residue weighing 2.8 Gm. (fraction I). The ethereal filtrate was extracted with 1% sodium hydroxide solution which produced a quantity of flocculent material, insoluble in both phases (fraction II). The sodium hydroxide solution yielded very little material upon acidification and extraction, hence, no further work was done. The ether solution was dried and concentrated to yield about 6.5 Gm. of a yellow residue (fraction III).

[1] Through the kindness of E. H. R. Jones, a small amount of this material was obtained for comparison.

Fraction II.—The flocculent material was suspended in water, acidified, and extracted with ether. The residue from the ether was recrystallized from acetone then chromatographed on alumina. The best melting point that could be obtained was 236–242°. Since the purity was doubtful, further work on this fraction was temporarily suspended.

Fraction I.—*Obliquol.*—The granular material was recrystallized from acetone to give needles, m. p. 188–190°, $[\alpha]_D^{25}$ +61° (c = 1.19, chloroform). Chromatography of the material on alumina did not improve these values. The name obliquol was assigned to this compound.

Anal.—Calcd. for $C_{30}H_{50}O_2$: C, 81.39; H, 11.38. Found: C, 81.72; H, 11.48.

Infrared analysis indicated absorption at 3350 cm.$^{-1}$ (hydroxyl), 1030 and 1048 cm.$^{-1}$ (two hydroxyls), 1410 cm.$^{-1}$ (primary hydroxyl), and no double bonds in the appropriate region. Ultraviolet absorption showed maxima at 244 mμ (E = 270) and 252 mμ (E = 215). The Liebermann-Burchard test gave a pale yellow color turning green, then orange. The Salkowski test gave a rust-colored ring, tetranitromethane gave a yellow color, and the Rosenheim test was negative.

Obliquol Diacetate.—Obliquol (500 mg.) was converted to the diacetate with acetic anhydride and fused sodium acetate. The product was recrystallized from ethanol-water to give lamina, m. p. 162–164°, $[\alpha]_D^{25}$ +39° (c = 1.39, chloroform).

Anal.—Calcd. for $C_{34}H_{54}O_4$: C, 77.52; H, 10.33. Found: C, 77.30; H, 10.33.

Obliquol Dibenzoate.—Obliquol (150 mg.) was converted to the dibenzoate with benzoyl chloride in pyridine and recrystallized from acetone-water after chromatography on alumina to give needles, m. p. 179–181°, $[\alpha]_D^{25}$ +75.3° (c = 1.13, chloroform).

Anal.—Calcd. for $C_{44}H_{58}O_4$: C, 81.19; H, 8.98. Found: C, 81.26; H, 8.97.

A quantity of the dibenzoate was hydrolyzed with 3% methanolic potassium hydroxide to give back obliquol, confirmed by the mixed melting point.

Obliquol Diacetate Tribromide.—Obliquol diacetate (100 mg.) in dry ether was treated with 2 ml. of a 10% bromine solution in glacial acetic acid. After removal of the solvents, the tan residue was recrystallized from methanol-water to give an amorphous substance, m. p. 97–99° (capillary).

Anal.—Calcd. for $C_{34}H_{52}Br_2O_4$: Br, 31.40. Found: Br, 32.10.

Perbenzoic Acid Titration of Obliquol Diacetate.—To 36 mg. of obliquol diacetate in 9 ml. of chloroform at 0° was added 5 ml. of 0.3 *N* perbenzoic acid in benzene at 0°. The reaction mixture was allowed to stand at 0° in the dark. At the end of twenty-four hours an aliquot was withdrawn and added to 25 ml. of water containing 1.5 Gm. potassium iodide and 1.5 ml. of glacial acetic acid. The liberated iodine was titrated with a standard sodium thiosulfate solution. A blank was similarly treated. The sample was found to have consumed the equivalent of 1.77 double bonds. After thirty-two hours the consumption was equivalent to 1.95 double bonds.

Hydrogenation-Dihydroobliquol Diacetate.—Obliquol diacetate (50 mg.) in ethyl acetate was hydrogenated over prereduced platinum oxide in a quantitative atmospheric pressure hydrogenator. The compound consumed the equivalent of one double bond. The ethyl acetate was removed and the residue recrystallized from ethanol-water to give plates, m.p. 165.5–167.5°, $[\alpha]_D^{25}$ +66.2° (c = 1.08, chloroform). A mixed melting point with obliquol diacetate gave a definite depression.

Anal.—Calcd. for $C_{34}H_{56}O_4$: C, 77.22; H, 10.67. Found: C, 77.61; H, 10.80.

Dihydroobliquol.—Dihydroobliquol diacetate (150 mg.) was hydrolyzed in 300 ml. of 3% methanolic

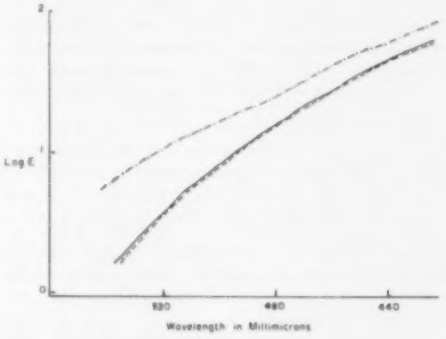


Fig. 1.—Tetranitromethane complex spectra for lanosterol ——, obliquol - - - -, and dihydroobliquol-·-·-

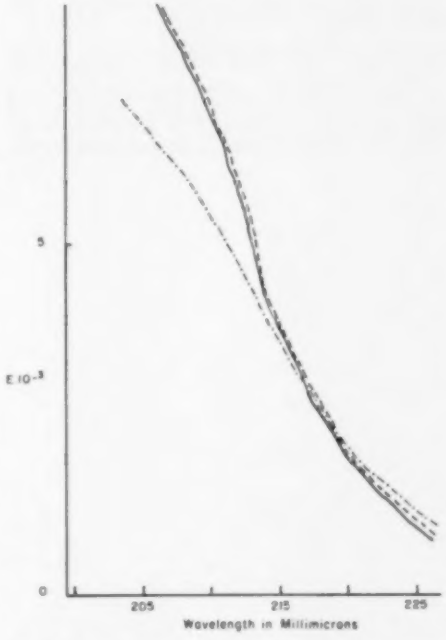


Fig. 2.—Ultraviolet spectra of lanosterol ——, obliquol - - - -, and dihydroobliquol-·-·-·-

potassium hydroxide. The product was recrystallized from methanol-water to give needles, m. p. 176–178°, $[\alpha]_D^{25}$ +56.8° (c = 0.99, chloroform).

Anal.—Calcd. for $C_{30}H_{52}O_3$: C, 81.02; H, 11.79. Found: C, 81.31; H, 11.80.

Dihydroobliquol gave a very faint yellow color with tetranitromethane.

Dihydroobliquol Dibenzoate.—Dihydroobliquol (80 mg.) was converted to the dibenzoate with benzoyl chloride in pyridine to give needles from acetone, m.p. 190–192°, $[\alpha]_D^{25}$ +67.5°(c = 1.43, chloroform).

Anal.—Calcd. for $C_{44}H_{60}O_5$: C, 80.93; H, 9.21. Found: C, 80.81; H, 9.04.

Perbenzoic Acid Titration of Dihydroobliquol Diacetate.—Dihydroobliquol diacetate (36 mg.) was allowed to react in the manner previously described with a benzene solution of perbenzoic acid at 0°. After twenty-four hours the equivalent of 0.96 double bond was consumed and after forty-eight hours this was raised to 1.03 double bond.

Fraction III.—The yellow residue from the ethereal solution (1 Gm.) was chromatographed on an alumina column. Hexane-benzene (1:1) eluted a fraction, IIIA, weighing about 300 mg. A second substance was eluted with pure benzene, fraction IIIB, weighing about 350 mg. This second fraction was recrystallized from acetone to give needles, m. p. 188–190°. A mixed melting point with obliquol gave no depression and the infrared spectra were identical.

Fraction IIIA.—*Lanosterol.*—This fraction was recrystallized several times from acetone-water to give needles, m. p. 137–139°. An acetate was prepared using acetic anhydride and fused sodium acetate and recrystallized from ethanol-water, m. p. 127–129°. A mixed melting point of IIIA and lanosterol gave no depression and the infrared spectra were identical.

Comparative Tetranitromethane Complex Spectra

Samples of obliquol (6.5 mg.), dihydroobliquol (2.5 mg.), and lanosterol (10 mg.) were placed in Beckman quartz cuvettes. To each was added 3.0 ml. of a 0.95 *M* solution of tetranitromethane solution in carbon tetrachloride. A fourth cuvette was filled with the reagent to serve as a blank. The absorption was measured from 380 to 550 mμ. The wavelength was plotted against extinction calculated from: $E = D/[C]\ [TNM].1$. See Fig. 1.

Comparative Ultraviolet Spectra

The ultraviolet spectra of obliquol, dihydroobliquol, and lanosterol were obtained in the 205 to 225 mμ region and the extinction coefficients plotted against wavelengths. See Fig. 2.

Tumor Inhibition Testing of Water Extract

Poria obliqua conks (50 Gm.) were extracted with water in a Waring Blendor, filtered, and evaporated to dryness *in vacuo* to yield 3.2 Gm. of scaly residue. This material was submitted to the Cancer Chemotherapy Section of the National Institutes of Health for testing against several tumors. The crude water extract was reported to have tumor inhibiting activity within acceptable limits for natural products against Sarcoma 180 and Carcinoma 755, but no activity against Leukemia 1210 below the toxic dose.

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Ketals and Hemithioketals of 1-Methyl-4-piperidone and Tropinone

By MATHIAS P. MERTES† and OLE GISVOLD

The preparation of ketals and hemithioketals of 1-methyl-4-piperidone and tropinone as potential postganglionic parasympathetic blocking agents has been undertaken. It is proposed that the relative activity of these rigid compounds will allow a more accurate description of the spatial arrangement in such a compound for optimal activity.

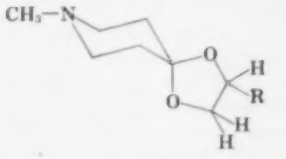
SINCE Pfeiffer's description of the sites of attachment of acetylcholine to a receptor surface (1) many investigations into the nature of this receptor site have dealt with its size, shape, and charge distribution. Lands (2) has noted several similarities in structure between acetylcholine and atropine and has proposed that the effects of cholinolytics arise from saturation of the acetylcholine receptor site by inactive molecules.

Several features of atropine have been discussed (3). Specifically noted was that the piperidine ring in atropine most probably exists in the chair form, however, according to Long (4) atropine acts in the boat form. Although it has not been demonstrated, atropine could convert to the boat form and be held in this manner at the receptor site by electrostatic attraction. Measurements of internuclear distances in atropine and pseudoatropine made on Dreiding-Stereomodels do not support this proposal. The internuclear distance of the nitrogen to the ether oxygen of the ester in the boat form of atropine being about 4 Å. is the same as the nitrogen to ether oxygen distance in the chair form of pseudoatropine (4 Å.). In addition, the hydroxymethyl and phenyl ring can be rotated to approximately the same spatial positions in both models. The benzilic acid ester of pseudotropine (equatorial) is reported to be one-third as active as a spasmolytic than tropylbenzilate (5). Therefore it appears unlikely that the latter would act in the boat form since the same distances would be presented in both isomers.[1]

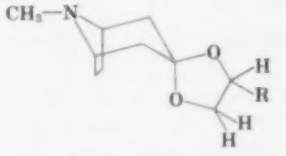
It is of interest that little attention has been directed at the optimal spatial arrangement of the groups in the tropic acid portion of atropine. Long (4) has incorporated the cationic head, the ester group, the hydroxyl, and the phenyl ring as possible points of attachment of atropine to the receptor site. It is possible that all of these groups are involved in attachment at the site but the distances cannot be specified in such a nonrigid system, nor can the spatial positions of these groups be defined. Thus the relative ease of movement of the groups in the tropic acid portion of the molecule prevents an accurate description of their spatial position for optimal activity.

The purpose of this investigation is the preparation of rigid spiro compounds containing some of the features of atropine in an attempt to evaluate the effect of the spatial position of the phenyl ring on cholinolytic activity. In addition, several of the compounds prepared contain two oxygens at the ring junction whereas the others contain a sulfur and an oxygen. The overall effect on cholinolytic activity of the sulfur substituted for one of the oxygens also will be of interest.

Ketals of 1-methyl-4-piperidone and tropinone were made with propylene glycol (I, II) and 3-phenylpropane-1,2-diol (III, IV). The latter glycol was prepared by solvolysis of 3-phenyl-1-chloro-2-propanol which was obtained by treating epichlorohydrin with phenylmagnesium bromide.



I R—CH_3
III R—$C_6H_5CH_2$



II R—CH_3
IV R—$C_6H_5CH_2$

The preparation of ketals of amino ketones, although by the usual methods of azeotropic distillation, in many cases required a longer period of

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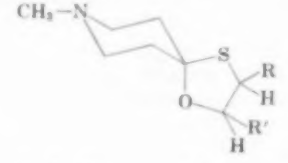
[1] The authors have not found specific reference to the relative spasmolytic activity of atropine vs. the equatorial ester.

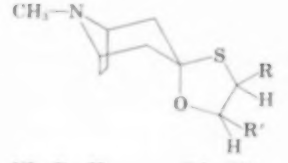
refluxing than normally encountered due to the insolubility of the amine salt. For the most part two catalysts were used: *p*-toluenesulfonic acid (method A) and dry hydrogen chloride (method B).

From studies made on Drieding-Stereomodels the conformation although not yet established is believed to be that wherein the R group is joined to the carbon adjacent to the equatorial oxygen.

Hemithioketals of the aminoketones were prepared utilizing 1-mercapto-2-propanol (V, VI), 2-mercapto-1-propanol (VII, VIII), 3-phenyl-1-mercapto-2-propanol (IX, X), and 3-phenyl-2-mercapto-1-propanol (XI, XII).

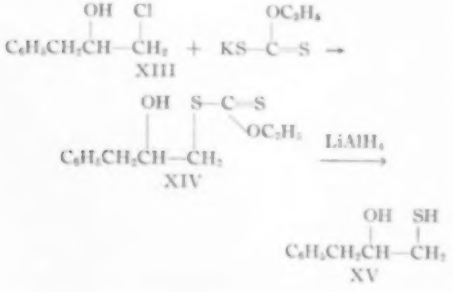


V	R—H	R′—CH_3
VII	R—CH_3	R′—H
IX	R—H	R′—$C_6H_5CH_2$
XI	R—$C_6H_5CH_2$	R′—H

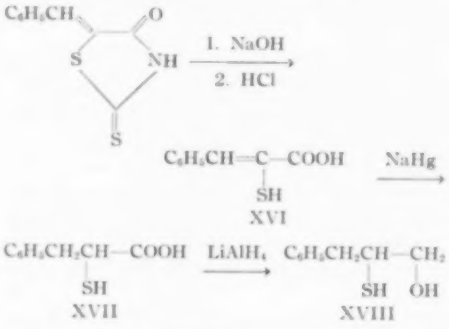


VI	R—H	R′—CH_3
VIII	R—CH_3	R′—H
X	R—H	R′—$C_6H_5CH_2$
XII	R—$C_6H_5CH_2$	R′—H

The thioglycols used in the preparation of the hemithioketals were prepared in the first two examples by reported procedures (6, 7). 3-Phenyl-1-mercapto-2-propanol (XV), previously reported by Djerassi (8), was prepared by reaction of 3-phenyl-1-chloro-2-propanol (XIII) with potassium ethylxanthate followed by reduction of the intermediate, 3-phenyl-1-ethylxanthate-2-propanol (XIV), with lithium aluminum hydride.

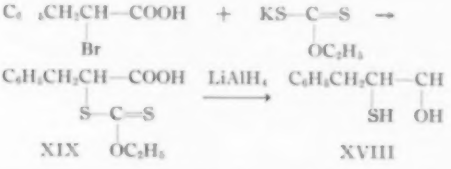


The isomer, 3-phenyl-2-mercapto-1-propanol (XVIII), was prepared by two procedures. The first was by the condensation of benzaldehyde with rhodanine and hydrolysis of the product, benzalrhodanine, to 2-mercaptocinnamic acid (XVI) (9). Direct reduction of this unsaturated acid with lithium aluminum hydride was unsuccessful. Stepwise reduction, first with sodium



amalgam to 3-phenyl-2-mercaptopropionic acid (XVII) and finally with lithium aluminum hydride gave the product (XVIII).

An alternate procedure was chosen in order to avoid contact with the vesicant, 2-mercaptocinnamic acid. This necessitated the preparation of 2-bromo-3-phenylpropionic acid (10), which was treated with potassium ethylxanthate to yield 2-ethylxanthate-3-phenylpropionic acid (XIX). Subsequent reduction with lithium



aluminum hydride gave the desired product, 3-phenyl-2-mercapto-1-propanol (XVIII).

The hemithioketals of the aminoketones were prepared by treating the aminoketone salt with the thioglycol in an appropriate solvent in the presence of an acid catalyst. Treatment of the aminoketone salts with 1-mercapto-2-propanol or 2-mercapto-1-propanol and dry hydrogen chloride (method B) followed by azeotropic distillation of the water formed in the reaction gave the respective hemithioketals (V-VIII).

The hemithioketals (IX, XI) of 1-methyl-4-piperidone with 3-phenyl-1-mercapto-2-propanol and its isomer, 3-phenyl-2-mercapto-1-propanol, were prepared in dioxane using zinc chloride as the catalyst (method C); *p*-toluene- sulfonic acid was found to be ineffective. Tropinone hydrochloride,

when refluxed in benzene with 3-phenyl-2-mercapto-1-propanol and dry hydrogen chloride (method B), gave the desired hemithioketal (XII).

Tropinone hydrochloride and 3-phenyl-1-mercapto-2-propanol failed to react when treated by azeotropic distillation using *p*-toluenesulfonic acid, dry hydrogen chloride, concentrated hydrochloric acid, 70% perchloric acid, trichloroacetic acid, ethereal hydrogen chloride, or boron trifluoride-etherate. The product (X) was eventually prepared by using an excess of boron trifluoride-etherate (method D) in an acetic acid solution of tropinone hydrochloride and 3-phenyl-1-mercapto-2-propanol.

From the size of sulfur atom and the probability that the ring converts to the least hindered form, the conformation of the hemithioketals of 1-methyl-4-piperidone is believed to exist mainly in the form wherein sulfur is equatorial and the oxygen is axial. In the tropinone hemithioketals additional interference of the axial substitutent with the hydrogens on the ethylene bridge leads to the proposal that sulfur is equatorial and the oxygen is axial. Previous reports (11, 12) in studies on ketosteroids have noted that 1-3 interference results in the sulfur assuming the equatorial position when possible.

The infrared spectrum of the ketals (I-IV) shows strong absorption in the region 1115-1099 cm.$^{-1}$ whereas that for the hemithioketals (V-XII) is at 1089-1071 cm.$^{-1}$ The latter agrees with Djerassi's assignment of 1075-1071 cm.$^{-1}$ as specific for hemithioketals (13). Another strong peak noted in the hemithioketals appeared in the region 1031-1022 cm.$^{-1}$

EXPERIMENTAL

All reported boiling points are uncorrected. The microanalyses were performed by the Department of Chemistry Microanalytical Laboratory, University of Minnesota, Minneapolis. The infrared spectra were obtained on a Beckman IR-5 either as Nujol mulls or carbon disulfide solutions.

3-Phenylpropane-1,2-diol.—A solution of 74 Gm. of sodium carbonate monohydrate (0.6 mole) in 500 ml. of water was placed in a 1-L. round bottom flask. One hundred grams of 1-chloro-3-phenyl-2-propanol (14) (0.59 mole) was added and the mixture refluxed for twenty-four hours. After cooling the oil was extracted with chloroform, dried with anhydrous sodium sulfate, and distilled using an air condenser (to avoid solidification in the condenser). The fraction boiling at 128–131°/0.75 mm. was collected yielding 75 Gm. (78%), n_D^{25} 1.5469, $\nu_{max.}^{CHCl_3}$ 1089 (secondary C—OH), 1032 cm.$^{-1}$ (primary C—OH). Reported boiling range (15) is 163–165°/15 mm.

3-Phenyl-1-mercapto-2-propanol.—An acetone solution (500 cc.) of 148 Gm. of 3-phenyl-1-chloro-2-propanol (14) (0.87 mole) and 161 Gm. of potassium ethylxanthate (1.0 mole) was refluxed for four hours and allowed to stand eighteen hours at room temperature. The potassium chloride formed in the reaction was removed by filtration and the filtrate evaporated to a thick oil. This oil was dissolved in 500 ml. of ether, washed twice with water, and dried overnight with anhydrous sodium sulfate.

A suspension of 50 Gm. of lithium aluminum hydride (95%, 1.25 moles) in 2 L. of anhydrous ether, previously dried over calcium hydride, was prepared in a 5-L., three-necked, round bottom flask fitted with a dropping funnel, stirrer, condenser, and a drying tube. The ether solution of the crude xanthate was added at a rate to maintain gentle refluxing. The entire addition required three hours. Refluxing was continued two hours and the mixture allowed to stand at room temperature for eighteen hours.

The excess lithium aluminum hydride was decomposed by the slow addition of ethyl acetate until refluxing ceased. Twenty-five per cent sulfuric acid then was added until the mixture separated into two layers. The ether layer was removed and the aqueous layer extracted twice with 200-ml. portions of ether. These combined ether extracts were quickly extracted with four 100-ml. portions of 15% sodium hydroxide. The sodium hydroxide extracts were drawn into 500 ml. of 25% sulfuric acid. The oily hemithioglycol separated and was extracted with four 300-ml. portions of ether, dried overnight with anhydrous sodium sulfate, and distilled. The product was collected at 106–110°/0.65 mm. yielding 74 Gm. (51%), n_D^{25} 1.5746, $\nu_{max.}^{CHCl_3}$ 2558 (CS—H), 1082 cm.$^{-1}$ (secondary C—OH), [reported (8) 84–87°/0.3 mm., n_D^{22} 1.5692].

Anal.—Calcd. for $C_9H_{12}OS$: C, 64.24; H, 7.19. Found: C, 63.68; H, 7.09.

The diacetate derivative was prepared by heating 5 Gm. of 3-phenyl-1-mercapto-2-propanol (0.03 mole), 2 Gm. of fused sodium acetate, and 20 ml. of acetic anhydride on a steam bath for one hour. After cooling the solution, it was poured into 100 ml. of ice water. The oily product was extracted with ether, washed with cold 10% sodium hydroxide, water, and dried with anhydrous sodium sulfate. After removal of the solvent by distillation the product was collected at 123–127°/0.2 mm. yielding 4.5 Gm. (60%), n_D^{25} 1.5329. A sample for analysis was redistilled and collected at 132°/0.45 mm.

Anal.—Calcd. for $C_{13}H_{16}O_2S$: C, 61.88; H, 6.39. Found: C, 61.71; H, 6.33.

2-Mercapto-3-phenylpropionic Acid.—This compound was prepared according to the procedure of Fischer (16) from 2-mercaptocinnamic acid (9) and sodium amalgam. The product was distilled and collected at 130–135°/0.3 mm. yielding 7 Gm. (46%), [reported (16) b. p. 184–187°/11–12 mm.]. This material should be handled carefully for it causes a very irritating and persistant rash.

3-Phenyl-2-mercapto-1-propanol.—This compound was prepared by two procedures.

(*a*). Forty grams of 2-mercapto-3-phenylpropionic acid (0.22 mole) in 500 ml. of anhydrous ether was added slowly to a suspension of 17 Gm. of lithium aluminum hydride (95%, 0.44 mole) in 1200 ml. of anhydrous ether. The addition required two hours and the suspension was refluxed an additional two hours. Decomposition of the excess lithium aluminum hydride with ethyl acetate followed by 25%

sulfuric acid gave separation into two layers. The ether layer was removed and the aqueous layer extracted with two 300-ml. portions of ether. The mercaptan was extracted from the ether with four 100-ml. portions of 15% sodium hydroxide and the latter immediately acidified with 500 ml. of 25% sulfuric acid. The resulting oil was extracted with ether, dried over anhydrous sodium sulfate, and distilled. The product, 28 Gm. (76%) was collected at 93–100°/0.2 mm. A redistilled sample for analysis was collected at 98–99°/0.1 mm., n_D^{25} 1.5728, $\nu_{max.}^{CS_2}$ 2558 (CS—H), 1031 cm.$^{-1}$ (primary C—OH).

Anal.—Calcd. for $C_9H_{12}OS$: C, 64.24; H, 7.19. Found: C, 63.98; H, 7.27.

(*b*) A solution of 6.7 Gm. of sodium bicarbonate (0.08 mole) and 14.5 Gm. of potassium ethylxanthate (0.09 mole) in 60 ml. of water was cooled to 15°; 18.4 Gm. of 2-bromo-3-phenylpropionic acid (10) (0.08 mole) was added slowly with stirring. After the addition was completed, the clear yellow solution was placed in the refrigerator at 10° for four days. The solution was acidified with 10 ml. of concentrated hydrochloric acid and extracted with ether. The ether extract was dried with anhydrous sodium sulfate and used as such in the subsequent reduction.

A suspension of 10 Gm. of lithium aluminum hydride (95%, 0.24 mole) in 400 ml. of anhydrous ether, previously dried over calcium hydride, was prepared in a 2-L., three-necked round bottom flask fitted with a dropping funnel, stirrer, condenser, and calcium chloride drying tube. An ether solution of the crude 2-ethylxanthate-3-phenylpropionic acid (theoretical amount 0.08 mole) was added slowly to the suspension over a sixty-minute period. The mixture was refluxed an additional two hours and allowed to stand at room temperature for eighteen hours. Decomposition of the excess lithium aluminum hydride was accomplished with ethyl acetate, followed by cautious addition of 25% sulfuric acid until the mixture separated into two clear layers. The ether layer was removed and the aqueous layer extracted with two 150-ml. portions of ether. The combined ether fractions then were extracted rapidly with four 50-ml. portions of 15% sodium hydroxide. The basic extracts were drawn into 400 ml. of 25% sulfuric acid. The oil that separated in the acid solution was extracted with ether, dried, and fractionated. The product, 3-phenyl-2-mercapto-1-propanol, was collected at 96–102°/0.27 mm. yielding 6 Gm. (46%). A sample for analysis was redistilled and collected at 88°/0.1 mm., n_D^{25} 1.5706.

Anal.—Calcd. for $C_9H_{12}OS$: C, 64.24; H, 7.19. Found: C, 64.53; H, 7.09.

1′,4-Dimethyl-spiro (1,3-Dioxolane-2,4′-piperidine) Hydrochloride (I).—*Method A.* Finely powdered 1-methyl-4-piperidone hydrochloride (17) (4.7 Gm., 0.032 mole) and 50 mg. of *p*-toluenesulfonic acid were added to 100 ml. of benzene in a 300-ml. round bottom flask equipped with a Dean-Stark separator and a reflux condenser. The suspension was heated to reflux for five minutes and 3 Gm. of propylene glycol (0.039 mole) was poured into the hot suspension. The refluxing was continued and after several minutes the amine hydrochloride melted, forming an oily layer on the bottom of the flask. After two hours of refluxing, the oil dissolved in the benzene. At this time 0.3 ml. of water had separated. Crystals gradually appeared on the sides of the flask and heating was stopped after three hours. The mixture was allowed to remain overnight at room temperature and filtered. The crystals were washed with benzene and dried, yielding 3.4 Gm. (51%). After several recrystallizations from ethanol-ether the product melted at 217–219°, $\nu_{max.}^{Nujol\ mull}$ 1149, 1133, 1104 (s), 1057 (s), 930 cm.$^{-1}$.

Anal.—Calcd. for $C_9H_{18}ClNO_2$: C, 52.04; H, 8.74. Found: C, 51.87; H, 8.69.

The free base was prepared by neutralization of 1 Gm. of the hydrochloride salt with a saturated solution of potassium carbonate. This was extracted with ether and dried over anhydrous sodium sulfate. Two milliliters of methyl iodide was added to the ether solution of the free base and after standing several hours the precipitated methiodide was filtered. Recrystallization from methanol-ether gave a white powder melting at 230–231°.

Anal.—Calcd. for $C_{10}H_{20}INO_2$: C, 28.55; H, 6.44. Found: C, 38.33; H, 6.40.

4-Benzyl-1′-methyl-spiro (1,3-Dioxolane-2,4′-piperidine) Hydrochloride (III).—This compound was prepared by method A from 7.4 Gm. of 1-methyl-4-piperidone hydrochloride (0.05 mole) and 7.6 Gm. of 3-phenylpropane-1,2-diol (0.05 mole). The product, 1.2 Gm. (8.5%), after several recrystallizations from methanol-ether melted with decomposition at 150–151°, $\nu_{max.}^{Nujol\ mull}$ 1101 (s), 1062, 932 cm.$^{-1}$.

Anal.—Calcd. for $C_{15}H_{22}ClNO_2$: C, 63.48; H, 7.81; N, 4.93. Found: C, 63.62; H, 7.72; N, 4.90.

The methiodide was prepared and recrystallized from methanol-ether; m. p. 191–193°.

Anal.—Calcd. for $C_{16}H_{24}INO_2$: C, 49.37; H, 6.21. Found: C, 49.38; H, 6.26.

4′-Benzyl-8-methyl-spiro (8-Azabicyclo-(3.2.1)-octane-3,2′-1′,3′-dioxolane) Hydrochloride (IV).—*Method B.*—A suspension of 5.0 Gm. of freshly prepared tropinone hydrochloride (18) (0.029 mole) and 7.6 Gm. of 3-phenylpropane-1,2-diol (0.05 mole) was prepared in 50 ml. of benzene and placed in a 200-ml. round bottom flask. The mixture was heated to reflux and dry hydrogen chloride bubbled into the hot solution for two minutes, effectively saturating the solution. The gas inlet tube was replaced by a Dean-Stark separator and condenser. After refluxing for one hour, more hydrogen chloride was bubbled into the mixture for one minute. The mixture was refluxed an additional two hours and during the last hour crystals began to appear. A total of 0.8 ml. of water separated. The mixture was cooled to room temperature and the product collected by filtration. Four recrystallizations from methanol-ether gave 5.1 Gm. (55%) of product melting at 238.5–239.5°, $\nu_{max.}^{Nujol\ mull}$ 1115 (s), 1062, 994 cm.$^{-1}$.

Anal.—Calcd. for $C_{17}H_{24}ClNO_2$: C, 65.90; H, 7.81. Found: C, 65.66; H, 7.78.

4′,8-Dimethyl-spiro (8-Azabicyclo-(3.2.1)-octane-3,2′-1′,3′-dioxolane) (II).—This compound was prepared by method B from 11 Gm. of freshly prepared tropinone *p*-toluenesulfonate (0.037 mole) and 7.6 Gm. of propylene glycol (0.1 mole). After neutralization of the benzene solution with a saturated solution of potassium carbonate, the benzene layer was dried over anhydrous sodium sulfate. The solvent was removed by distillation and the residue fractionated. The product was collected at 61–64°/0.18 mm. and weighed 4 Gm. (53%). A sample for analysis was distilled and collected at 76–77°/1.0 mm., n_D^{25} 1.4809, $\nu_{max.}^{CHCl_3}$ 1143, 1099 (s), 1070 cm.$^{-1}$.

Anal.—Calcd. for $C_{11}H_{19}NO_2$: C, 66.95; H, 9.71. Found: C, 67.23; H, 9.62.

1′,5-Dimethyl-spiro (1,3-Oxathiolane-2,4′-piperidine) Hydrochloride (V).—This compound was prepared by method B from 5 Gm. of 1-methyl-4-piperidone hydrochloride (0.034 mole) and 4 Gm. of 1-mercapto-2-propanol (0.043 mole) in 50 ml. of benzene. The product was filtered yielding 5.5 Gm. (72%). After six recrystallizations from methanol-ether the product melted at 203–205°, $\nu_{max.}^{Nujol\ mull}$ 1095, 1070 (s), 1047, 988, 970 cm.$^{-1}$.

Anal.—Calcd. for $C_9H_{18}ClNOS$: C, 48.31; H, 8.10. Found: C, 48.58; H, 8.27.

The methiodide of the base was prepared and recrystallized three times from methanol-ether; melting point 222–224°.

Anal.—Calcd. for $C_{10}H_{20}INOS$: C, 36.48; H, 6.12. Found: C, 36.31; H, 6.22.

5′,8-Dimethyl-spiro (8-Azabicyclo-(3.2.1)-octane-3,2′-1′,3′-oxathiolane) Hydrochloride (VI).—This compound was prepared by method B from 5 Gm. of tropinone hydrochloride (0.029 mole) and 3.7 Gm. of 1-mercapto-2-propanol (0.04 mole). The product was collected by filtration and after four recrystallizations from methanol-ether gave 1.73 Gm. (24%) melting with decomposition at 260–261°, $\nu_{max.}^{Nujol\ mull}$ 1089 (s), 1070, 1049 (s), 1025, 937 cm.$^{-1}$.

Anal.—Calcd. for $C_{11}H_{20}ClNOS$: C, 52.89; H, 8.07. Found: C, 53.01; H, 7.88.

1′,4-Dimethyl-spiro (1,3-Oxathiolane-2,4′-piperidine) Hydrochloride (VII).—This compound was prepared by method B from 5 Gm. of 1-methyl-4-piperidone hydrochloride (0.034 mole) and 4 Gm. of 2-mercapto-1-propanol (0.043 mole). The crystalline product was removed by filtration yielding 4 Gm. of product which after recrystallization from methanol-ether melted at 204–206°, $\nu_{max.}^{Nujol\ mull}$ 1074 (s), 1060, 1043, 1031, 985 cm.$^{-1}$.

Anal.—Calcd. for $C_9H_{18}ClNOS$: C, 48.31; H, 8.10. Found: C, 48.55; H, 8.10.

The methiodide was prepared and recrystallized from methanol-ether, m. p. 249–251°.

Anal.—Calcd. for $C_{10}H_{20}INOS$: C, 36.48; H, 6.12. Found: C, 36.32; H, 6.07.

4′,8-Dimethyl-spiro (8-Azabicyclo-(3.2.1)-octane-3,2′-1′,3′-oxathiolane) Hydrochloride (VIII).—This compound was prepared by method B using 5 Gm. of tropinone hydrochloride (0.029 mole) and 4 Gm. of 2-mercapto-1-propanol (0.043 mole). The product was collected and recrystallized six times from methanol-ether yielding 0.92 Gm. (13%) melting at 263–264°, $\nu_{max.}^{Nujol\ mull}$ 1099 (s), 1082 (s), 1046, 1025, 974 (s) cm.$^{-1}$.

Anal.—Calcd. for $C_{11}H_{20}ClNOS$: C, 52.89; H, 8.07. Found: C, 53.18, H, 8.11.

5-Benzyl-1 -methyl-spiro (1,3-Oxathiolane-2,4′-piperidine) Methiodide (IX).—*Method C.*—A suspension of 6 Gm. of 1-methyl-4-piperidone hydrochloride (0.04 mole), 10 Gm. of 3-phenyl-1-mercapto-2-propanol (0.06 mole), 10 Gm. of anhydrous sodium sulfate, and 10 Gm. of freshly fused and powdered zinc chloride was prepared in 75 ml. of dioxane. This mixture was shaken and complete solution of everything except the sodium sulfate was apparent after one hour. After standing three days at room temperature, the mixture was alkalinized with 50 ml. of 28% ammonium hydroxide solution. The mixture was extracted three times with 50-ml. portions of chloroform, the chloroform extracts washed with water, and finally evaporated. The thick oily residue was redissolved in ether, dried over anhydrous sodium sulfate, and distilled. The yellow oil boiling at 148–158°/0.6 mm. was collected yielding 4.1 Gm. of product (45%). A methiodide derivative was prepared and recrystallized three times from methanol-ether; melting point 230–232°, $\nu_{max.}^{Nujol\ mull}$ 1074 (s), 1031, 982, 949, 913 cm.$^{-1}$.

Anal.—Calcd. for $C_{16}H_{24}INOS$: C, 47.41; H, 5.97. Found: C, 47.14; H, 5.96.

5′-Benzyl-8-methyl-spiro (8-Azabicyclo-(3.2.1)-octane-3,2′-1′,3′-oxathiolane) Methiodide (X).—*Method D.*—Three grams of tropinone hydrochloride (0.017 mole) was dissolved in 15 ml. of glacial acetic acid with the aid of heat. Six grams of 3-phenyl-1-mercapto-2-propanol (0.036 mole) was added and the clear yellow solution cooled to room temperature. Boron trifluoride-etherate (8 ml., 45% BF_3, 0.052 mole) was added rapidly and the solution shaken vigorously for fifteen minutes, during which time the flask was cooled occasionally to room temperature. The acetic acid was removed by vacuum distillation from a water bath at 85°. The residue was cooled to 10° and washed with 30 ml. of cold 15% sodium hydroxide. The oily suspension was extracted three times with 30-ml. portions of ether and finally with 20 ml. of chloroform. The combined organic extracts were washed twice with 50 ml. of water, dried, and evaporated. The oily residue was redissolved in ether, filtered to remove insoluble material, dried, and again evaporated. A methiodide derivative of the residue was made and recrystallized five times from methanol-ether. The product (0.41 Gm., 5.6%) melted at 204–205°, $\nu_{max.}^{Nujol\ mull}$ 1074, 1053, 1037 (s), 1022 cm.$^{-1}$.

Anal.—Calcd. for $C_{16}H_{24}INOS.H_2O$: C, 48.11; H, 6.28; N, 3.11. Found: C, 48.13; H, 6.07; N, 2.86.

4-Benzyl-1′-methyl-spiro (1,3-Oxathiolane-2,4′-piperidine) Methiodide (XI).—This compound was prepared according to method C from 6 Gm. of 1-methyl-4-piperidone hydrochloride (0.04 mole), 10 Gm. of 3-phenyl-2-mercapto-1-propanol (0.06 mole), 10 Gm. of sodium sulfate, and 10 Gm. of zinc chloride in 75 ml. of dioxane. After neutralization of the solution and partial purification, the crude product was an oil that was subjected to fractional distillation. The fraction boiling at 138–145°/0.3 mm. was collected and weighed 4.5 Gm. (49%). The methiodide that was prepared and recrystallized four times from methanol-ether melted at 193–193.5°.

Anal.—Calcd. for $C_{16}H_{24}INOS$: C, 47.41; H, 5.97. Found: C, 47.15; H, 6.12.

The citrate derivative of the free base was prepared by adding a saturated ether solution of citric acid to an ether solution of the base. The product, recrystallized four times from methanol-ether melted at 129–131°, $\nu_{max.}^{Nujol\ mull}$ 1084 (s), 1056, 1031, 977, 945 cm.$^{-1}$.

Anal.—Calcd. for $C_{21}H_{29}NO_8S$: C, 55.37; H, 6.42. Found: C, 55.15; H, 6.30.

4′-Benzyl-8-methyl-spiro (8-Azabicyclo-(3.2.1)-octane-3,2′-1′,3′-oxathiolane) Hydrochloride (XII).—This compound was prepared by method B from 5 Gm. of tropinone hydrochloride (0.029 mole) and 6.7 Gm. of 3-phenyl-1-mercapto-2-propanol (0.04 mole) in 50 ml. of benzene. The product was filtered and found to be impure. Fractional crystal-

lization from methanol-ether gave 0.8 Gm. (6.9%) of product melting at 250–251°, $\nu_{max.}^{Nujol\ mull}$ 1078 (s), 1057, 1029, 1004, 973 cm.$^{-1}$.

Anal.—Calcd. for $C_{17}H_{35}ClNOS$: C, 62.65; H, 7.42. Found: C, 62.83; H, 7.30.

SUMMARY

1. The isomeric thioglycols 3-phenyl-1-mercapto-2-propanol and 3-phenyl-2-mercapto-1-propanol have been synthesized, the latter by two methods.

2. The preparation of ketals of 1-methyl-4-piperidone and tropinone with proplyene glycol and 3-phenylpropane-1,2-diol has been described.

3. Hemithioketals of the two aminoketones have been prepared from 1-mercapto-2-propanol, 3-phenyl-1-mercapto-2-propanol, 2-mercapto-1-propanol, and 3-phenyl-2-mercapto-1-propanol.

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Comparative Laboratory Evaluation of Antiseborrheic Dermatitis Preparations

By LEO GREENBERG

Twenty-four pharmaceutical formulations suggested for the control of seborrheic dermatitis were tested for antibacterial activity, antifungal activity, and cellular toxicity. Under the test conditions, the highest lethality to microbial cells was demonstrated by povidone iodine, mercurials, and 4,6-dichlorophenol. Of all of the agents tested, only bis-laurel trimethylammonium polythionate, colloidal sulfur, coal tar, and povidone iodine were less toxic to mammalian cells than to microbial cells.

SEBORRHEIC DERMATITIS may well be the most common single disease entity of civilized man, with a national incidence in America of approximately 70 per cent (1). It is an affliction of the upper layer of the skin, most commonly of the scalp, and may range in severity from an unnoticed crusting with occasional itching to unbearably painful eczematous patches. Even in mild cases, the concomitant symptoms of dandruff and hair loss pose serious social and esthetic problems. Its diagnosis is almost self-evident, and its symptoms and complications have been extensively catalogued in every textbook of dermatology.

Despite this situation, however, its cause is almost completely obscure (2), although there is general agreemen[t] among specialists in the field that both excessi[ve] activity of the sebaceous glands and the presence of specific microorganisms are involved (3). Since the etiology of the disease is in doubt, it is not surprising that remedies by the score have been introduced into medical practice, some of real and some of apparently imaginary value (4).

In most cases, the evaluation of such remedies has been a subjective clinical one, and rarely have two therapeutic agents been compared under standard conditions. Therefore, a comparative laboratory evaluation was undertaken to obtain information concerning the relative usefulness of the most commonly available preparations. Although over fifty commercial trademarked preparations were collected, duplications in basic formulas reduced the number actually evaluated to twenty-four. These were tested for antibacterial activity, antifungal activity, and cellular toxicity.

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MATERIALS AND METHODS

Commercial antiseborrheic dermatitis preparations were purchased from local pharmacies. Identification of the active ingredients and the concentrations thereof were made from the label or product information furnished by the manufacturer. Dilutions were made with sterile saline in Wasserman tubes and kept well mixed.

The organisms chosen as test substrates include those listed as having been most frequently associated with diseased scalps (5). Representative species were purchased from the American Type Culture Collection, maintained on appropriate media, and transplanted weekly. For more luxuriant growth, *Corynebacterium acnes* was grown under reduced oxygen tension. As needed, a small loopful of surface growth was transferred to a tube of sterile FDA broth and incubated at 37° for eight hours. One loopful of this broth was then inoculated into fresh sterile FDA broth for eighteen hours. Preliminary study showed that such tubes for each species contained approximately equal numbers of organisms regardless of the size of the initial inoculum.

Bactericidal values were determined by pipetting 0.2 ml. of broth culture of the test organism into sterile Wasserman tubes and adding 1.0 ml. of the appropriate dilution of the preparation. The tubes were then kept well mixed at 37° for exactly ten minutes. At that time, a 4-mm. loopful of the mixture was removed and transferred aseptically to a tube containing 2.0 ml. sterile broth. To reduce or eliminate the bacteriostatic effect of those compounds known to demonstrate this phenomenon, 5% sodium thiosulfate, 2% polysorbate 80, 0.5% agar, and 1% thioglycollate were incorporated in the broth tubes in the case of povidone iodine, hexachlorophene, quaternary ammonium, and mercury disinfectants, respectively. Broth tubes were then incubated for forty-eight hours (anaerobically in the case of *C. acnes*) at 37° and growth or no growth, as evidenced by turbidity recorded. Tubes showing growth were checked by Gram stain to confirm the characteristic organism. All determinations were made in duplicate.

Toxicity to living mammalian cells was tested on rat leukocytes obtained from healthy young male animals, following a technique previously reported from our laboratory (6), the end point being deter-

TABLE I.—BACTERICIDAL DILUTIONS AND CELLULAR TOXICITY VALUES FOR THE EVALUATED PREPARATIONS

Product	Active Ingredients	Germicidal Dilution[a]					Leukocidal Dilution	B/L Ratio
		M. flavus[b]	*C. acnes*[c]	*P. vulgaris*[d]	*M. aureus*[e]	*P. ovale*[f]		
1	Povidone iodine	1:5120	1:640	1:160	1:1280	1:640	1:1000	0.8
2	Mercury (0.45%); phenol (0.5%); cresol (0.75%); soap	1:2560	1:2560	1:1280	1:640	1:10	1:10000	15.6
3	4,6-Dichlorophenol	1:1280	1:320	1:640	1:160	1:320	1:500	3.1
4	Alkyl dimethyl benzyl ammonium chloride; alkyl isoquinolinium halide	1:640	1:1280	1:160	1:320	1:160	1:1000	3.1
5	Mercuric iodide (1%); soap	1:640	1:320	1:160	1:320	1:80	1:12000	37.5
6	Entsufon; cholesterols; hexachlorophene (3%)	1:160	1:640	1:160	1:20	1:10	1:300	15.0
7	Selenium disulfide	1:320	1:160	1:80	1:160	1:40	1:400	2.5
8	4,6-Dichlorophenol (1%); sulfur (2%); salicylic acid (2%)	1:160	1:320	1:80	1:5	1:160	1:200	40.0
9	Bis-lauryl trimethylammonium polythionate; tetradecylamine N-lauroyl sarcosine; ethanol	1:320	1:80	1:160	1:10	1:2	1:2	0.2
10	Sulfur, 4,6-dichlorophenol	1:2	1:160	1:320	1:5	1:80	1:100	20.0
11	Crude irradiated coal tar	1:320	1:80	1:10	1:80	1:2	1:40	0.5
12	Sulfur (2%); salicylic acid (2%); hexachlorophene (1%)	1:40	1:40	1:80	1:5	1:5	1:700	140.0
13	Dihydroxy-dichloro-diphenyl methane; benzalkonium chloride; ethanol	1:80	1:2	[g]	1:5	[g]	1:30	6.0
14	Benzethonium chloride; N-trichloromethyl-mercapto-4-cyclohexene-1,2-dicarboximide; ethanol	1:40	[g]	1:5	1:2	[g]	1:100	50.0
15	Methyl benzethonium chloride	1:10	1:5	1:5	1:2	1:10	1:70	35.0
16	Tetrathionate; pentathionate; zirconium oxide (2%)	1:10	1:2	1:10	1:5	[g]	1:100	20.0
17	Colloidal sulfur	1:10	1:2	[g]	1:5	1:5	1:2	0.4
18	Sulfur; zinc sulfide; salicylanilide	1:10	1:5	1:2	[g]	[g]	1:10	...
19	Sulfacetamide (10%)	1:10	1:2	[g]	1:2	[g]	1:10	5.0
20	Cadmium sulfide (1%)	[g]	[g]	1:2	[g]	1:10	1:60	...
21	Tannic acid; tinctures of cantharides, benzoin, nutgall; ethanol; glycerin	1:5	1:2	1:2	[g]	[g]	1:10	...
22	Cerapon-C; tyrothricin; parabens; propylene glycol	1:5	1:2	[g]	[g]	[g]	1:5	...
23	Salicylic acid (2%); allantoin (0.2%); 4,6-dichlorophenol	1:2	1:5	[g]	[g]	[g]	1:5	...
24	Entsufon; cholesterols	[g]	[g]	[g]	[g]	[g]	1:20	...

[a] Minimum effective concentration of commercial preparation. [b] *Micrococcus flavus* ATCC 400. [c] *Corynebacterium acnes* ATCC 6919. [d] *Proteus vulgaris* ATCC 8427. [e] *Micrococcus pyogenes* var. *aureus* ATCC 6538. [f] *Pityrosporum ovale* ATCC 12078. [g] Full strength ineffective.

mined by the presence or absence of phagocytic ability toward a standard culture of *Micrococcus aureus*.

RESULTS AND DISCUSSION

Bactericidal dilutions and cellular toxicity values for each of the 24 preparations evaluated are listed in Table I. For convenience in comparing results, the preparations are arranged in decreasing order of activity. This was determined for each product by computing the mean effective dilution from the five end points obtained against the five test organisms. Since it was patently impossible to prepare all possible dilutions, the actual end point for any determination may be somewhat below that actually listed.

Inspection of the data reveals a high order of germicidal activity for povidone iodine, mercurials, and 4,6-dichlorophenol. In general, quaternary ammonium compounds and hexachlorophene rank moderately high in this regard, although it is known that their primary effect is bacteristatic rather than bactericidal. Sulfur compounds and preparations tend to rank relatively low, as do salts of cadmium and zirconium.

Many of the preparations tested were less effective against staphylococci (column 4) than against the other test bacteria, which would seem to be an eloquent testimonial to the hardiness of this ubiquitous organism.

The data on *Pityrosporum ovale* reveals the low order of sensitivity which this common, yeast-like organism displays to most antiseptic agents. Its susceptibility to povidone iodine and to 4,6-dichlorophenol is correlated with the wide spectrum of activity displayed by compounds of these groups.

It has been previously pointed out (6) that white blood cells represent an excellent and readily available substrate for the measurement of chemical toxicity to mammalian cells. Since the toxicity of antiseborrheic dermatitis preparations is of considerable concern, the leukocidal dilution for each of the products tested was determined. These data constitute column 6 of the table, and the sensitivity of leukocytes to many compounds, especially mercurials, is evident.

Column 7 indicates the bactericide/leukocide ratio of each product, using the *M. aureus* value, and furnished a simple toxicity index. A B/L ratio value of more than 1 indicates a higher toxicity to mammalian cells than to bacteria, while a B/L value of less than 1 indicates the converse. Theoretically, the lower the value, the safer the preparation or compound for routine use. In practice, such determinations can only be a guide, since leukocytes may differ from skin cells in sensitivity to chemical compounds.

It is noteworthy that only bis-lauryl trimethylammonium polythionate, colloidal sulfur, coal tar, and povidone iodine of the agents tested were less toxic to leukocytes than to bacteria.

SUMMARY

1. Twenty-four selected formulations for the treatment of seborrheic dermatitis have been tested for germicidal, fungicidal, and toxic properties.

2. Under the test conditions, povidone iodine, mercurials, and 4,6-dichlorophenol evidenced the highest antibacterial and antifungal activity.

3. Of the preparations tested, only bis-lauryl trimethylammonium polythionate, colloidal sulfur, coal tar, and povidone iodine were more toxic to microorganisms than to mammalian cells.

4. Of the preparations tested, povidone iodine most closely met the test criteria of a high order of lethality to microbial cells and a low order of toxicity to mammalian cells.

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Interaction of Carrageenan and Other Hydrocolloids with Alkaloids I

Precipitation Studies

By HORACE D. GRAHAM and LINNIE B. THOMAS†

Carrageenan, other natural plant hydrocolloids, and the synthetic products carboxymethylcellulose and Kelcoloid O are precipitated by alkaloids of the quinoline, isoquinoline, and tropane classes and by tubocurarine, a double tetrahydroisoquinoline alkaloid. The interaction, as exemplified by the carrageenan-cinchonine sulfate reaction, is highly temperature-sensitive, is suppressed by salts when they are present above a certain "tolerance level," and is diminished at low pH values. Generally, the sulfated plant hydrocolloids are more readily precipitated than the uronic acid type hydrocolloids. Comparative precipitabilities based on a "coined" standard which has been dubbed the "alkaloid-hydrocolloid precipitability index" reflect the practical and fundamental significance of such interactions in pharmaceutical or other formulations.

ONE IMPORTANT CHARACTERISTIC of the polyanionic plant hydrocolloid, carrageenan, is its interaction, with or without concomitant precipitation, with cationic substances such as dyes, quaternary ammonium compounds, proteins, and some alkaloids. This polyelectrolyte is widely used in pharmaceutical formulations, many of which may contain alkaloids or alkaloid-like ingredients. The efficacy of such alkaloids could be greatly influenced by their interaction with the hydrocolloid. Therefore, a study of such interactions should provide information of highly fundamental (1) as well as practical significance. This paper summarizes some of such studies and incorporates comparative investigations on other natural hydrocolloids and some synthetic products which are being used or are of potential value as emulsifiers and stabilizers in pharmaceutical and other products. The absorbance of the suspensions resulting from the reaction of the hydrocolloids and the alkaloids was used as a measure of the interaction. Several variables such as the temperature at which the reaction was carried out, the pH of the reaction medium, the influence of the reaction time, and the effect of added inorganic salts and sodium benzoate were investigated.

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EXPERIMENTAL

Materials.—*Alkaloids.*—Quinine hydrochloride, Merck and Co., Inc., Rahway, N. J., 0.050 *M*. Cinchonine sulfate, Nutritional Biochemicals Corp., Cleveland, Ohio, 0.030 *M*. Tubocurarine chloride, Nutritional Biochemicals Corp., Cleveland, Ohio, 0.008 *M*. Atropine sulfate, Mann Research Laboratories, Inc., New York 6, N. Y., 0.007 *M*. Hyoscyamine hydrochloride, Mann Research Laboratories, Inc., New York 6, N. Y., 0.060 *M*. Hydrastine hydrochloride, Inland Alkaloids, Inc., Tipton, Indiana, 0.025 *M*.

Hydrocolloids.—These are listed in Table I. The moisture content of each sample was determined by heating the samples to constant weight in a vacuum oven at 80°. Inorganic salts C. P. were used without further purification. Sodium benzoate U.S.P. powder, (J. T. Baker Chemical Co., Phillipsburg, N. J.) was used.

Equipment.—Constant temperature water bath, Coleman universal spectrophotometer, model 14. Pyrex, 30-ml. capped test tubes. Beckman pH meter, model G. Vacuum oven, Precision Scientific Co.

Procedure.—For all preliminary experiments and for studies on the influence of variables, the cinchonine sulfate-carrageenan (SeaKem type 3) interaction was used.

Dialysis of Gum Samples.—Gum samples of concentrations 0.05–0.1% were prepared by dusting weighed amounts of the particular gums onto cold distilled water. Complete solution was achieved by gently heating the solutions with constant stirring, if necessary. The solutions were then placed in 36/32 dialysis membranes and dialyzed against distilled water for thirty-six hours at 4° with six changes of distilled water. All dialyzed solutions were stored at 4° and fresh samples were prepared every seven days. Hypnean was extracted from the seaweed according to the procedure given by Smith and Montgomery (2).

Preparation of the Alkaloid Solutions.—Solutions of concentrations indicated in Table II were prepared either in 20% ethyl alcohol or distilled water.

Turbidity Measurements.—Quantities of carrageenan or the other hydrocolloids ranging from 0–1.0

Table I.—Comparative Precipitability of Hydrocolloids by Alkaloids[a]

Hydrocolloid	Quinine HCl HPI[c]	Cinchonine Sulfate HPI[c]	Tubocurarine Chloride HPI[c]	Hydrastine HCl HPI[c]
Group 1				
Carrageenan (SeaKem type 402)	0.06	0.05	0.15	0.04
Carrageenan (SeaKem type 5)	0.09	0.07	0.11	0.07
Carrageenan (SeaKem type 7)	0.06	0.05	0.15	0.05
Furcellaran	0.07	20.69	0.51	...
Sodium kappa carrageenan	0.07	0.14	0.06	0.07
Sodium lambda carrageenan	0.05	0.10	0.12	0.04
Gigartina acicularis (extract)	0.06	0.66	1.40	0.04
Gigartina pistillata (extract)	0.06	0.47	0.18	0.04
Eucheuma cottonii (extract)	0.10	1.68	0.06	0.08
Eucheuma spinosum (extract)	0.07	0.76	0.11	0.04
Agar	4.10	6.3	[b]	3.00
Hypnean	2.10	11.10	0.20	31.25
Iridophycan	0.09	0.30	1.60	0.15
Group 2				
Gum arabic (acacia)	50.0	[b]	[b]	...
Gum karaya	4.00	55.00	[b]	10.0
Pectin	2.60	[b]	[b]	15.0
Gum tragacanth	10.20	18.00	[b]	...
Gum ghatti	13.30	18.74	[b]	12.0
Group 3				
Kelmar	0.10	66.0	[b]	12.0
Kelco gel HV	0.10	0.097	[b]	10.0
Kelcoloid O	2.20	75.0	[b]	60.0
Sodium carboxymethylcellulose	1.00	[b]	[b]	...
Gelatin	[b]	[b]	[b]	...
Gum guar	10.50	24.0	6.0	5.45
Locust bean gum	13.30	40.0	[b]	...

[a] Atropine sulfate and hyoscyamine, at the concentrations used, gave no precipitation. [b] No precipitation. [c] Alkaloid-hydrocolloid precipitability index.

Table II.—Quantitative Reaction of Carrageenan (Seakem Type 3) with Alkaloids at 30°

Carrageenan, mg.	Quinine Hydrochloride (0.050 *M*), Absorbance	Cinchonine Sulfate (0.030 *M*), Absorbance	Tubocurarine Chloride (0.008 *M*), Absorbance	Hydrastine Hydrochloride (0.025 *M*), Absorbance
0.1	0.097	...	...	0.070
0.2	0.197	0.161	...	0.150
0.3	0.301	...	...	0.220
0.4	0.403	0.323	...	0.290
0.5	0.488	...	0.112	0.370
0.6	0.585	0.444	...	0.430
0.8	...	0.602	...	0.580
1.0	...	0.745	0.215	0.710
1.5	...	...	0.303	...
2.0	...	...	0.409	...

mg. were placed in consecutive test tubes and, where necessary, the volumes made up to 6 ml. with distilled water. Four milliliters of the alkaloid solution was added to each test tube with gentle shaking, and the mixture incubated for thirty minutes at 30° in a constant temperature water bath. At the end of this period, the absorbance of each tube was measured at 400 mμ with a Coleman Universal spectrophotometer, model 14. The blank in each case consisted of 4 ml. of the particular alkaloid solution diluted to 10 ml. with distilled water.

Factors Influencing the Reaction.—To assess the influence of variables, the turbidity developed by reacting 0.6 mg. of carrageenan (SeaKem type 3) with cinchonine sulfate according to the general procedure was determined for periods or under conditions shown in Table III.

Table III.—Influence of Variables and Added Salts on the Interaction of Carrageenan Type 3 with Cinchonine Sulfate

Influence of Variables		Influence of Added Salts	
Variable	Absorbance, 400 mμ	Salt Added as Chloride	Tolerance Level (*M*)
Temperature, °C.		Monovalent	
30	0.457	K^+	5×10^{-4}
35	0.450	Li^+	2.5×10^{-4}
40	0.292	Na^+	5×10^{-4}
45	0.158	NH_4^+	5×10^{-4}
Reaction Time, min.		Divalent	
5	0.387	Ca^{++}	1.25×10^{-4}
10	0.420	Mg^{++}	5×10^{-4}
15	0.422	Ba^{++}	5×10^{-4}
30	0.444	Cd^{++}	1×10^{-3}
45	0.446	Cu^{++}	1×10^{-4}
60	0.444	Hg^{++}	1×10^{-4}
120	0.448	Mn^{++}	1×10^{-3}
pH		Ni^{++}	1×10^{-4}
3.2	0.109	Sr^{++}	1×10^{-4}
4.6	0.190	Zn^{++}	1×10^{-4}
4.8	0.240	Trivalent	
5.1	0.280	Al^{+++}	1×10^{-5}
5.3	0.337	Fe^{+++}	1×10^{-5}
5.5	0.337		
5.8	0.409	Organic	
6.1	0.420	$C_2H_3COO^-$	1×10^{-4}
6.3	0.414		
6.5	0.380		
6.6	0.367		

Studies on pH were done by adding varying amounts of 0.1 *N* acetic acid and 0.01 *N* sodium

hydroxide to the test tubes, mixing the contents well, and determining the absorbance according to the general procedure. The final pH was determined with a Beckman model G pH meter. Since salts interfere with the interaction (Table III), it was not possible to obtain meaningful results in buffered systems.

For investigating the influence of added inorganic salts and sodium benzoate, as listed in Table III, on the reaction, varying amounts of the salts were added to the reaction tubes and the turbidity which developed was measured. That maximum final concentration (the concentration of salt in the 10 ml. of reaction medium) of salt which did not cause a significant difference in the absorbance as compared to a control tube to which no salt was added, has been labeled the "tolerance level."

Quantitative Relationship.—The ready precipitability of the hydrocolloids by several of the alkaloids used allowed the establishment of quantitative relationships thus permitting a systematic comparison of the precipitability of the various hydrocolloids by the alkaloids.

To establish such quantitative relationships, varying amounts of carrageenan (SeaKem type 3) were reacted with alkaloids at 30° for thirty minutes and the absorbance of the products determined, as outlined under the section on Turbidity Measurements of the general procedure. Table II summarizes the results. For the alkaloids used, the following logarithmic least squares equations describe the relationships:

$$X_{\text{quinine hydrochloride (Q.HCl)}} = 1.99926 - \text{Log } \% T/0.9986$$

$$X_{\text{cinchonine sulfate (C.s.)}} = 1.97183 - \text{Log } \% T/0.69475$$

$$X_{\text{hydrastine hydrochloride (HYDR.HCl)}} = 1.99309 - \text{Log } \% T/0.66485$$

$$X_{\text{tubocurarine chloride (T.c.)}} = 1.94287 - \text{Log } \%T/0.14998$$

In all cases, X = mg. carrageenan type 3 reacting with the particular alkaloid.

Comparative Precipitability of Hydrocolloids.—In order to compare their susceptibility to precipitation, 0.05–6.0 mg. (dry weight basis) of the hydrocolloids listed in Table I were reacted with various alkaloids and the absorbance of each system measured, as outlined under the section on Turbidity Measurements of the general procedure. Since some solutions were appreciably viscous, accompanying control tubes were included by substituting 4 ml. of 20% ethyl alcohol or distilled water for the alkaloid solution. By substituting the per cent transmission of the test system in the carrageenan-alkaloid logarithmic least squares equation and making the correction for the blank, the number of milligrams of carrageenan (SeaKem type 3) which would give the same turbidity as that amount of any other hydrocolloid used was obtained. By calculation, an arbitrary comparative value termed the "alkaloid hydrocolloid-precipitability index (AHPI)" was then obtained. This has been defined as the number of milligrams of any hydrocolloid which, when reacted with a particular alkaloid, will give the same turbidity (absorbance) as the product of the reaction of 0.1 mg. of dialyzed carrageenan (SeaKem type 3) and the said alkaloid (under the experimental conditions employed). This arbitrary measure will differ for each hydrocolloid and for each alkaloid precipitant. Designations such as cinchonine sulfate-hydrocolloid-precipitability index (cinchonine sulfate HPI) etc., therefore, arise. The value also depends upon the hydrocolloid-alkaloid system used as the standard and, for any similar comparison, a standard curve or equation for the hydrocolloid-alkaloid primary must be established and absorbances for definite concentrations of the unknowns determined in a similar manner.

As the AHPI increases, the precipitability of the hydrocolloid by a particular alkaloid decreases, hence direct comparisons can be made. Interactions with atropine sulfate and hyoscyamine sulfate, at the concentrations used, produced no measurable turbidity; therefore, standard curves and equations were not established for comparisons.

RESULTS AND DISCUSSION

Primarily, the interaction between the anionic polyelectrolyte, carrageenan, and the alkaloid is considered to be mainly ionic in nature. However, as seen from Table III, the intense thermal sensitivity of the system suggests that, although ion-ion interaction (electrostatic) force plays a part in the combination of carrageenan and other such hydrocolloids with alkaloids, other interactions such as ion-dipole, dipole-dipole, and van der Waals forces apparently come into play. Such interactions are known to occur between proteins and other molecules (3, 4). Possibly, hydrogen bonding is also a contributing binding force as suggested by studies on the interaction of fibrinogen with dextran sulfate (4). Ehrenpreis and Kellock (5) obtained evidence that in the precipitation of the sulfated mucopolysaccharide, chondroitin sulfate, with local anesthetics and curare, binding in solution does not reflect complex formation by purely coulombic forces.

Under the experimental conditions employed, at 30° the maximum density of the interaction product occurred after fifteen minutes and, within the limits of experimental error, remained constant for as long as two hours, the maximum incubation period employed.

At low pH values, the interaction was hindered due, apparently, to inhibition of ionization of the polyanionic hydrocolloid (3, 6). This inhibition would most likely be more severe with the uronic acid type hydrocolloids (carboxyl group) than with the sulfated hydrocolloids (sulfate group) since, at the pH levels investigated, ionization of the sulfate group would not be as drastically impeded. In the alkaline range, the alkaloids precipitate in the absence of any hydrocolloid hence, this range is not experimentally feasible. Minimum variations in absorbance occurred between pH 5.8–6.3. A systematic check on the final pH of all interaction systems showed that they all fell within the pH range 5.8–6.2. The pH of the hydrocolloid-hydrastine hydrochloride reaction mixture was 4.4–4.5 and was studied as such.

Precipitation of carrageenan by cinchonine sulfate declined as the concentration of added salt increased. By analogy, all the other similar systems used can be considered to respond in the same way. This declination in precipitability may be due to neutralization of ionizable groups in the hydrocolloids by

counterions in the mixture. Table III shows that, generally, the trend of inhibition by the cations is as follows: trivalent > divalent > monovalent. Ba^{++} and Mg^{++} are exceptions among the divalent forms. The benzoate ion, commonly incorporated into pharmaceutical formulations, exhibited an inhibitive capacity equivalent to that of most of the divalent cations; it probably competed with the anionic polymer for the combining groups of the alkaloid.

With respect to precipitability by the various alkaloids, the hydrocolloids listed in Table I can be arranged into three broad groups on the basis of their reactivities: *Group* 1.—This group comprises the sulfated plant hydrocolloids and, as reflected by their precipitability indexes, are the most reactive natural plant hydrocolloids with those alkaloids indicated in Table I.

Group 2.—This group comprises the uronic acid type natural plant hydrocolloids. In terms of reactivity, these are the next most reactive.

Group 3.—This group comprises those natural or synthetic hydrocolloids which do not fall under either group 1 or group 2. Their reactivities follow no consistent pattern.

Sodium carboxymethylcellulose is precipitated by quinine hydrochloride but not by the other alkaloids used. Gelatin, a protein, was not precipitated by any of the alkaloids despite the fact that the final pH of the solution (6.18) was such that the protein would be negatively charged (7). Kelco Gel HV, a sodium alginate product, exhibited precipitability equal to that of the sulfated polysaccharides with quinine hydrochloride and cinchonine sulfate. Kelmar, an algin product, behaved similarly when reacted with quinine hydrochloride. Precipitation of gum guar and locust bean gum by quinine hydrochloride and cinchonine sulfate suggests the possible role of hydroxyl groups in ion-dipole, dipole-dipole interactions and the probable involvement of van der Waals forces (8).

It is of interest to note that, except for the interaction with tubocurarine chloride, the precipitability of lambda carrageenan was greater than that of kappa carrageenan. Application of the "significance of difference test" to the data on the precipitation of the two fractions showed that the observed differences were significant at the 0.05, 0.005, and 0.10% levels for quinine hydrochloride, cinchonine sulfate, and hydrastine hydrochloride, respectively. The standard deviations were found to be 0.003, 0.0005, and 0.003 for quinine hydrochloride, cinchonine sulfate, and hydrastine hydrochloride, respectively. This is consistent with chemical data which shows that kappa carrageenan has significantly less ester sulfate than lambda carrageenan (2, 9). The anomalous behavior with tubocurarine chloride is not readily explicable.

Absence of precipitation with certain alkaloids is not indicative of nonreaction. However, such subtle interactions can be determined only by techniques such as equilibrium dialysis (10,11), data for which will be reported in another communication. Stoloff (1) noted that a negatively charged macromolecule does not necessarily precipitate carrageenan and urged a study of the relative structures of precipitants and nonprecipitants. Data obtained here suggest that such selective precipitation holds true for polyanionic hydrocolloids, both related and unrelated to carrageenan. Reported absence of complex formation between chondroitin sulfate, procaine, and tetracaine, despite extremely high concentrations of anionic groups of the mucopolysaccharide (5), support the findings here. Kennon and Higuchi (12) discuss the prevalence of incorporation of polyelectrolytes in pharmaceutical systems.

Important considerations cited include drug insolubilization, appearance, stability, and prolongation of action. Prolongation of the time of therapeutic efficacy through "timed" release of medication was pointed out, the digitoxin-CMC and amphetamine-caffeine combinations being examples. Data presented here demonstrate clearly that incorporation of hydrocolloids in pharmaceutical formulations could possibly influence the efficacy of any alkaloid or alkaloidal agent present. Such influence would vary depending on the hydrocolloid employed.

The results recorded in Table I, indicate that the structure of the alkaloid is of great importance in the precipitation of hydrocolloids. Comparatively, the tropane alkaloids represented by atropine and hyoscyamine are evidently highly nonreactive at low concentrations. Tubocurarine chloride, a quaternary ammonium compound (13), readily precipitated the group 1 hydrocolloids except agar, a supposedly comparatively lightly sulfated polysaccharide. Failure of this alkaloid to precipitate the group 2 and group 3 hydrocolloids indicates some specificity or preferential reaction of the anionic group. Since quinine hydrochloride and cinchonine sulfate, two closely related alkaloids, precipitated almost all the hydrocolloids the quinoline alkaloids are, apparently, very reactive. Similarly, hydrastine hydrochloride, representing the isoquinoline group of alkaloids, proved very reactive.

Comprehensively, the results indicate not merely the interaction between hydrocolloids and alkaloids, but may be extended to other biochemically important sulfated compounds like the mucopolysaccharides and carboxyl compounds like uronic acids and other chemicals which may be present in pharmaceuticals (14).

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Isosteric Mercurial Diuretics

By WILLIAM R. JONES†, DONALD B. MEYERS, and JOHN W. MARTIN

Four isosteric mercurial diuretics were synthesized and studied to ascertain the difference between the amide and ester grouping on diuretic activity. A method of synthesis and evidence for an anhydro zwitterion type of structure are presented.

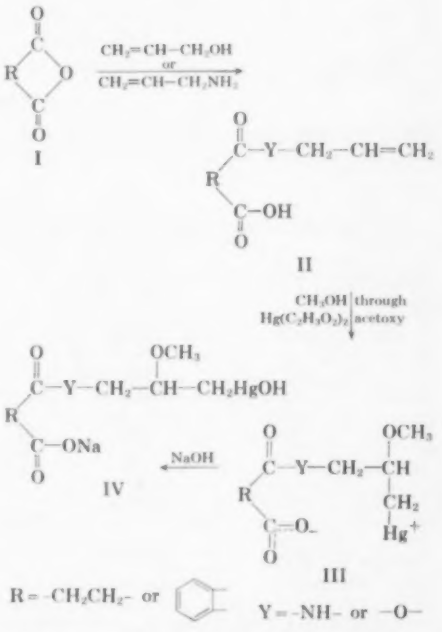
FOR MANY years the mercurial diuretics in common use have been characterized as mercurated monoallyl amides of dicarboxylic acids. Recently, however, a number of diuretics have been synthesized which do not conform to this pattern. The structure of some of these compounds indicate that the amide linkage may not be essential to diuretic activity.

In 1928 a compound containing an ester linkage in lieu of the amide grouping, β-hydroxy-γ-hydroxymercuripropyl succinate, was tested for diuretic properties and found to be inactive (1). Mercurated 8-allyloxycaffeine, containing an ether type linkage, was also reported to be diuretically inactive (2). Apparently the reports on these two compounds were responsible for the statement (3) that, "Mercurated allyl alcohol and its simple derivatives possess little, if any, diuretic activity and ethers and esters of mercurated allyl alcohol are also generally weak."

Since it was felt that an investigation of the relative influence of the ester *versus* the amide linkage in diuretic mercurials would be a contribution to an understanding of the structure-activity relationship, structural representatives of aliphatic and aromatic amide diuretics and their oxygen analogs were prepared and evaluated for activity and toxicity.

DISCUSSION

The monoallyl esters and amides of succinic and phthalic acids (II) were prepared by reacting the respective acid anhydrides (I) with allyl alcohol or allyl amine (4–9). By this procedure pure mono derivatives were obtained. These compounds were then mercurated by reacting them with a solution of mercuric acetate in methyl alcohol. The initial acetoxy derivative was not isolated but immediately hydrolyzed. A soluble sodium salt (IV) was obtained upon the addition of alkali. The reaction may be illustrated as follows:



Of the possible compounds that could be produced by the sequence of reactions employed, step II to III, e. g., acetoxy, hydroxy, cyclic, or anhydro ionic, the results of elemental analysis clearly indicated that a cyclic or anhydro ionic form was the one obtained. As early as 1930, Moller (10) on the basis of carbon, hydrogen, and mercury analyses proposed that mersalyl was excreted in the urine in the form of a cyclic compound. Later Rowland, *et al.* (11), postulated a cyclic structure when their elemental analytical results did not agree with the open chain structure they expected to obtain. Much more recently (12) an anhydro zwitterion-type structure has been proposed for products resulting from alkoxymercuration of olefinic compounds. These structures were substantiated by infrared data.

In the present investigation similar evidence for the anhydro zwitterion structure was obtained by a study of the infrared spectra. The mull spectrum for the mercurated allyl succinate, Fig. 1, shows an ester carbonyl at 5.75 μ and the carboxylate ion bands at 6.2 and 7.4 μ. In Fig. 2 for the mercurated allyl phthalate, the ester carbonyl is strong at 5.82 μ. The carboxylate absorption at 6.09 μ is unusually narrow and at a shorter wavelength, but is not within a range to be considered a covalent ester-like group. Specific assignment cannot be made in

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Carbon-hydrogen analysis by Weiler and Strauss, Oxford, England.
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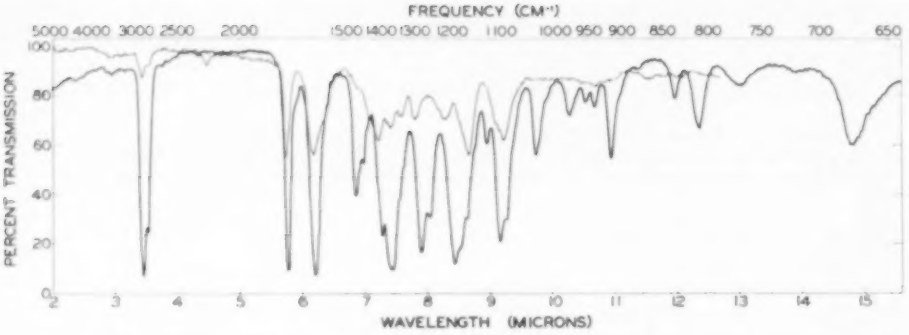

Fig. 1.—Infrared spectrum for the mercurated allyl succinate. Narrow line is saturated solution in $CDCl_3$, wide line is mineral oil mull.

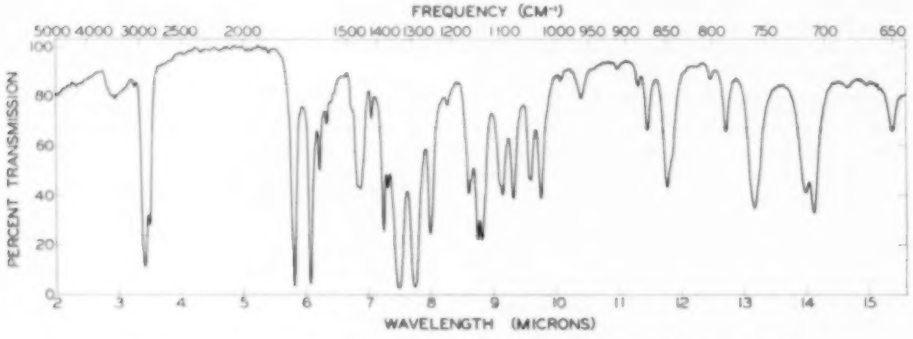

Fig. 2.—Infrared spectrum for the mercurated allyl phthalate, mineral oil mull.

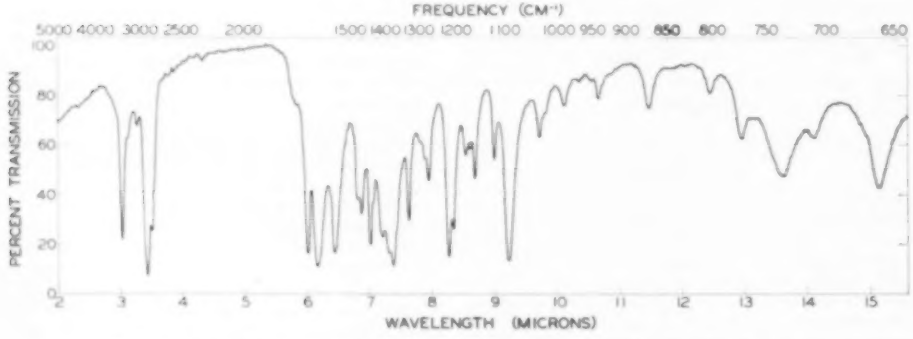

Fig. 3.—Infrared spectrum for the mercurated allyl succinamide, mineral oil mull.

the 7–8 μ range, but the two broader, more intense bands are probably carboxylate and ester. The broad band of moderately low intensity for hydroxyl is probably due to water or methanol in the crystal. In Fig. 3 for the mercurated allyl succinamide the mull spectrum shows bands at 3.03, 6.02, and 6.45 μ for the amide and bands at 6.16 and 7.3 μ (broad structure) for the carboxylate ion. Absence of a band for the carboxyl group in both III and IV further indicates the salt between the carboxyl and the mercuric ion. In Fig. 4 assignment of bands among the aromatic, amide, and carboxylate cannot be clearly made, but since there is no intense band for the covalent ester-like group the mercury is considered bound ionically to the carboxylate.

Pharmacology.—Results of the pharmacological investigation (13) showed that intravenously all of the compounds tested were as active as N-(3-carboxy-methylmercaptomercuri-2-methoxypropyl)-α-

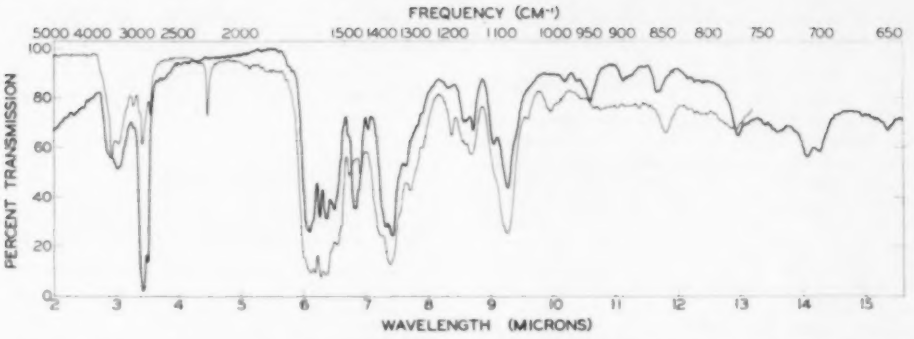

Fig. 4.—Infrared spectrum for the mercurated allyl phthalamide. Narrow line is sample in $CDCl_3$. The band at 4.5 μ arises from an intensification of the deuterochloroform C-D stretching by hydrogen bonding with the solute. Wide line is the mineral oil mull.

camphoramate.[1] With the exception of the mercurated allyl ester of phthalic acid all of the compounds exhibited significant sodium diuresis when administered orally to dogs. On the basis of the LD_{50} determinations the mercurated allyl amide of phthalic acid appears to be of the same order of toxicity as the mercurial diuretics in common use, whereas the other compounds were more toxic. It was shown that the amides are not necessarily more potent or less toxic than corresponding esters.

EXPERIMENTAL

Mercurated Allyl Succinate (Anhydro-3-hydroxymercuri-2-methoxypropyl Hydrogen Succinate).—Twenty grams (0.2 mole) of succinic anhydride was refluxed with 42 ml. (0.6 mole) of allyl alcohol for approximately one hour. The resulting liquid was vacuum distilled and the fraction collected between 140–143° at 5 mm.; yield 11.2 Gm., 35%.

Anal.—Calcd. for $C_4H_8O_2$-COOH: neut. equiv., 158.2. Found: 161.8.

To a hot solution of 40 Gm. (0.125 mole) of mercuric acetate in 700 ml. of methyl alcohol was added 20 Gm. (0.126 mole) of the ester dissolved in an equal volume of boiling methyl alcohol. The precipitate which began to form immediately was removed by filtration after the solution had been allowed to stand several hours. The precipitate was boiled with distilled water for two hours and then washed with diluted methyl alcohol; yield, 37.5 Gm., 76%, of a white powder which melted at 187–188°.

Anal.[2]—Calcd. for $C_8H_{10}HgO_5$: C, 24.68; H, 3.08; Hg, 51.62. Found: C, 25.25; H, 3.30; Hg, 51.40.

Mercurated Allyl Phthalate (Anhydro-3-hydroxymercuri-2-methoxypropyl Hydrogen Phthalate).—Fifty grams (0.337 mole) of phthalic anhydride was mixed with 50 ml. (1.103 mole) of allyl alcohol and heated for fifteen minutes. Upon cooling the small amount of precipitate was filtered off. The oily filtrate was washed with distilled water and then dried. It decomposed on attempted vacuum distillation. Yield of dry oil 34 Gm., 49%.

Anal.—Calcd. for $C_{10}H_9O_2$-COOH; neut. equiv. 206.0. Found: 207.0.

Twenty grams (0.097 mole) of the ester was dissolved in 50 ml. of methyl alcohol. To this was added a solution of 30.8 Gm. (0.097 mole) of mercuric acetate dissolved in 60 ml. of methyl alcohol. A white precipitate formed almost immediately; yield, 40.0 Gm., 94.5%; m.p., 230–235° (decompn.).

Anal.—Calcd. for $C_{12}H_{14}HgO_5$: C, 32.98; H, 2.76; Hg, 45.94. Found: C, 32.26; H, 3.02; Hg, 45.48.

(The low analysis is probably due to associated water or methyl alcohol as seen on the infrared spectra.)

Mercurated Allyl Succinamide (Anhydro-3-hydroxymercuri-2-methoxypropyl Hydrogen Succinamide).—To 100 Gm. (1.0 mole) of succinic anhydride suspended in 400 ml. of ethyl acetate was added slowly, with stirring, a solution containing 75 ml. of allyl amine (1.0 mole) in an equal volume of ethyl acetate. The solution was cooled in an ice bath as the allyl amine was added. After concentrating the reaction mixture to about one-fourth the original volume, a precipitate gradually formed. The crude product was recrystallized from hot chloroform; yield, 54.0 Gm., 34%; m. p., 91–92°.

Anal.—Calcd. for $C_4H_{10}NO$-COOH: neut. equiv., 157.2. Found: 156.7.

Twenty grams (0.62 mole) of mercuric acetate was dissolved by heating in a solution of 350 ml. of methyl alcohol containing 10 drops of nitric acid. To this hot solution was added 15 Gm. (0.095 mole) of allyl succinamide. The solution was heated for thirty minutes. After standing, a white crystalline precipitate was obtained, m. p. 193–196°.

Anal.—Calcd. for $C_8H_{13}HgNO_4$: C, 24.76; H, 3.35; Hg, 51.49. Found: C, 24.89; H, 3.44; Hg, 51.15.

Mercurated Allyl Phthalamide (Anhydro-3-hydroxymercuri-2-methoxypropyl Hydrogen Phthalamide).—To 20.0 Gm. (0.135 mole) of phthalic anhydride suspended in 200 ml. of ethyl acetate was added a solution of 10 ml. of allyl amine (0.122 mole) mixed with an equal volume of ethyl acetate. The amine solution was added slowly and with constant swirling. After standing, the precipitate

[1] Thiomerin (Wyeth), used as the sodium salt.
[2] Mercury analysis by a modification of U. S. P. method for mersalyl according to Theimer, E. E., and Arnow, P., This Journal, 44, 381(1955).

which formed was recrystallized from a solution of hot chloroform; yield, 48.0 Gm., 84%; m. p. 110–111°.

Anal.—Calcd. for $C_{19}H_{17}NO$-COOH: neut. equiv., 295.2. Found: 201.0.

Ten grams (0.048 mole) of the allyl phthalamide was dissolved in 50 ml. of methyl alcohol. A solution of mercuric acetate was prepared by dissolving 15.35 Gm. (0.048 mole) of the acetate in 300 ml. of hot methyl alcohol containing 0.5 ml. of nitric acid. The hot acetate solution was filtered into the hot amide solution with the formation of a white precipitate. The product was recrystallized from dilute methyl alcohol; yield, 18.0 Gm., 85%; m. p., 152–154°.

Anal.—Calcd. for $C_{19}H_{19}HgNO_5$: C, 33.08; H, 2.98; Hg, 46.05. Found: C, 33.11; H, 3.39; Hg, 45.05.

SUMMARY

1. Synthesis of the required intermediates for ultimate mercuration, such as monoallyl succinate, monoallyl phthalate, N-allylsuccinamic acid, and N-allylphthalamic acid is described.

2. Mercuration of the above compounds to form anhydro zwitterion structures is described.

3. The structures of these anhydro derivatives is established by elemental analysis and infrared data.

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Ion Exchange Separation and Colorimetric Determination of Phenylephrine in Pharmaceutical Products

By CLARK A. KELLY and MORRIS E. AUERBACH

Since the introduction of phenylephrine, it has been used in combination with many other drugs. With some formulations it has been found useful to isolate the drug via a cationic exchange resin before determining the intact phenylephrine by the usual colorimetric procedures (coupling with diazotized *p*-nitroaniline, or reacting with Millon's reagent when catecholamines are present).

IN THE DECADE since Auerbach (1) described the azo colorimetric determination of phenylephrine hydrochloride U. S. P. (*l*-1-(*m*-hydroxyphenyl)-2-methylaminoethanol hydrochloride), the number of formulations in which this sympathomimetic is used have increased enormously. In most cases the presence of other active constituents has complicated the analysis for phenylephrine, particularly in stability testing. Methods involving paper chromatography, spectrophotofluorometry, ultraviolet spectrophotometry, bromination, and a number of colorimetric procedures (2–14) have been used, but are limited in most cases to simple phenylephrine preparations. The important step is then the quantitative separation of the phenylephrine from substances which interfere with its colorimetric determination. By the use of a cationic exchange resin (nuclear sulfonic type) separation from acidic and neutral substances is accomplished, and at the same time the selectivity of the intact phenylephrine assay is greatly increased. If the side chain were lost, or its basicity significantly lessened, the remaining molecule would not be held by the resin and so would be discarded with the water washes. The intact phenylephrine held by the resin is eluted with acid along with any basic material that may be eluted at the stated acid strength. Almost the only compounds which cannot be separated from phenylephrine by ion exchange,

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and which cause serious interference, are the catecholamines. In such cases, Millon's reagent, which is selective for mono-phenols, is used.

Cationic exchange resins have been used in this laboratory with good success in the separation of phenylephrine from many other drugs. Another laboratory (15) has applied a similar technique but elutes the phenylephrine from the resin with 0.1 *M* sodium hydroxide instead of acid. In special cases this may be useful, but it is well to remember that an alkaline medium favors oxidative destruction of the phenolic group, and might thus be responsible for erratic assays.

Vincent *et al.* (16), have used a variety of ion exchange resins in the separation of sympathomimetic amines. They reported, and this laboratory confirms, that Amberlite IRA-400 (a strongly basic anion exchanger) holds the phenylephrine so firmly that it is not eluted. With Amberlite IRC-50 (a weakly acidic cation exchanger) phenylephrine is not held quantitatively. Amberlite IR-45 (a weakly basic anion exchanger) was used successfully in their work. The phenylephrine is then determined through titration of its basic nitrogen. In this procedure, both the separation and assay depend upon the basic function, thus no information results as to the qualitative status of the phenolic group. The questions raised by Schriftman (6) in regard to determining intact phenylephrine in complex formulations during stability testing are met by the dual nature of the recommended procedure: (*a*) separation depending on the intact basic side chain (*b*) subsequent assay depending upon intact hydroxyphenyl function.

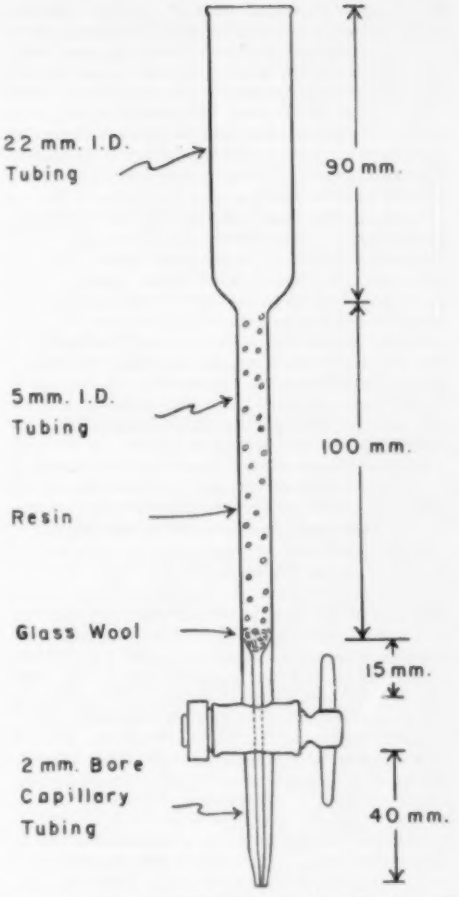

Fig. 1.—Ion exchange column for isolation of phenylephrine.

EXPERIMENTAL

Apparatus.—Glass column, see Fig. 1. The stopcock can be of glass or teflon; less "freezing" occurs with the latter.

Water bath, a 600-ml. beaker with a stainless steel screen support resting on the bottom.

Any suitable photocolorimeter, capable of selecting light bands at about 420 mμ and at about 500 mμ.

Reagents.—Cationic exchange resin in the hydrogen form, Dowex 50-X-1, 50–100 mesh or its equivalent. To be assured that the resin is free of any foreign matter which might interfere in the colorimetric procedures it is washed as follows: mix with a magnetic stirrer 100 Gm. resin in a 2 L. beaker with 500 ml. 3 *M* hydrochloric acid for fifteen minutes. Repeat washes twice more, discarding each acid wash. Wash the resin free of acid (pH tape color remains neutral). Store resin in distilled water until needed. Enough resin, about 2 Gm., is added in the form of a slurry to the glass column which has a small loose glass wool plug resting on the constriction above the stopcock. The resin height is 100 mm. which fills the column just to the reservoir portion of the column. The reservoir capacity is about 25 ml.

Hydrochloric acid, 0.5 *M*.

Nitric acid, 1 *M*.

Sodium hydroxide, 1 *M*.

Sodium hydroxide solution, 10%.

Borax (sodium tetraborate decahydrate) solution, 5%.

Diazo solution. Dissolve 30 mg. recrystallized *p*-nitroaniline[1] in 2 ml. dilute (6 *M*) hydrochloric acid. Chill in ice bath, add 0.5 ml. of 7% sodium nitrite solution. After two minutes add 100 ml. ice cold water and 1 ml. 4% sulfamic acid solution. Mix, and keep in the ice bath until needed. Even when cold, this reagent should not be used if more than three hours old.

Millon's reagent. Add 2 ml. triple distilled mercury to a 125-ml. conical flask. With swirling add

[1] Dissolve 10 Gm. of the *p*-nitroaniline in as little of hot 95% ethanol as possible, filter, let cool slowly, and then keep in an ice bath for at least an hour. Filter again. Dry the yellow crystals at 60° overnight. The melting range of the purified compound is 147°–149° corrected.

20 ml. concentrated (16 *M*) nitric acid and allow to stand for ten minutes in a well ventilated hood. Add 35 ml. water and mix. Add by pipet 10% sodium hydroxide solution until a permanent turbidity results (about 3 ml.). Add 5 ml. dilute (3 *M*) nitric acid. Keep this clear solution stoppered. Do not use after the first day of preparation. Millon's reagent reacts readily with protein matter (skin in particular) so great care should be used in handling it at all times.

Standard Solution.—Weigh exactly 100 mg. phenylephrine hydrochloride (U. S. P. grade) and transfer to a 100-ml. volumetric flask. Dissolve and make to volume with water (solution A). By appropriate dilution with water prepare the desired working standard.

For the Azo method.—Pipet 10 ml. of solution A to a 100-ml. volumetric flask and dilute to volume with water (solution B). Pipet 10 ml. of solution B to a 100-ml. volumetric flask and dilute to volume with water. This last dilution contains 10 mcg. phenylephrine hydrochloride per ml.

For the Millon method.—Pipet 5 ml. of solution A to a 100-ml. volumetric flask and dilute to volume with 1 *M* nitric acid. This last dilution contains 50 mcg. phenylephrine hydrochloride per ml.

Sample solutions.—Solutions or aqueous extracts of phenylephrine should be of such concentrations that not more than a 5-ml. aliquot containing a minimum of 250 mcg. phenylephrine hydrochloride for the azo method, and not more than a 25 ml. aliquot containing a minimum of 1.25 mg. for the Millon method is added quantitatively to the resin column. If larger aliquots are required, the washing step will involve more water than described for the isolation step.

An example for a tablet containing 5 mg. phenylephrine hydrochloride is as follows: determine the average weight of 20 tablets. Grind to a fine powder. Transfer a weight of the powder equivalent to one average tablet to a 250-ml. glass-stoppered conical flask. Add exactly 100 ml. water and shake mechanically for ten minutes. Filter through Whatman No. 1 paper or its equivalent, discarding the first 25 ml. Take a 5-ml. aliquot of the clear filtrate by pipet and add to the resin column reservoir.

Azo Method (No Catecholamines Present).—An example is given below, in a formulation containing N - acetyl - *p* - aminophenol, phenylephrine hydrochloride, thenyldiamine hydrochloride, and caffeine. Pass an aqueous solution containing at least 250 mcg. phenylephrine hydrochloride in a volume not greater than 5 ml. through the resin column at a flow rate of 1 to 2 ml. per minute. Wash the column with 5-ml. portions of water at the same flow rate, draining each wash to the top of the resin column, until 25 ml. have been collected in a graduated cylinder. Discard these washes. Place a 25-ml. volumetric flask under the column and elute the phenylephrine with five 5-ml. portions of 0.5 *M* hydrochloric acid collecting exactly 25 ml. of eluate. Mix, and transfer by pipet 2 ml. to a test tube graduated accurately at the 10 ml. volume. To an ordinary test tube add a 2-ml. aliquot and one drop of phenolphthalein test solution U. S. P. From a 5 or 10 ml. semimicro buret graduated in 0.05 ml. titrate this 2-ml. aliquot with 1 *M* sodium hydroxide to the phenolphthalein end point (first permanent red). To the aliquot in the graduated test tube add the same amount of 1 *M* sodium hydroxide less 0.05 ml. Great care must be taken at this step to assure valid results.

To this nearly neutral solution and to a series of similar tubes containing standard phenylephrine hydrochloride (0, 10, 20, 30 mcg.) add water so that each tube contains 5–6 ml. solution. Add 2 ml. of the 5% borax solution and mix. Add 0.5 ml. of the fresh cold diazo solution, mix, and let stand for ten minutes. Add 1 ml. 10% sodium hydroxide. Dilute to the 10 ml. mark with water and mix. Read in a photocolorimeter at 500 mμ, setting the instrument with the reagent blank. Calculate the amount of phenylephrine hydrochloride present from the standard values obtained at the same time the sample is analyzed.

Millon Method (Catecholamines Present).—An example is a formulation containing 0.125% phenylephrine hydrochloride and 0.5% N-isopropylethyl norepinephrine hydrochloride. Pass an aqueous solution containing at least 1.25 mg. phenylephrine hydrochloride in a volume not greater than 25 ml. through the resin column at a flow rate of 1 to 2 ml. per minute. Wash the column with 5-ml. portions of water, draining each wash to the top of the resin column, until 100 ml. has been collected in a graduated cylinder. Discard these washes. Place a 25-ml. volumetric flask under the column and elute the phenylephrine with five 5-ml. portions of 1 *M* nitric acid, collecting exactly 25 ml. of the eluate. Mix, and transfer by pipet 3 ml. to a test tube graduated accurately at the 10 ml. volume. Prepare a series of similar tubes containing standard phenylephrine hydrochloride (0, 100, 150, 200 mcg.). Adjust the volume in each tube to 5 ml. with 1 *M* nitric acid. Add 4 ml. Millon's reagent to each tube. Mix, and keep in a boiling water bath for fifteen minutes. Allow the tubes to cool for at least thirty minutes at room temperature. Adjust the volume in each tube to 10 ml. with 1 *M* nitric acid. Mix, and read the clear solutions in a photocolorimeter at 420 mμ, setting the instrument with the reagent blank. Calculate the amount of phenylephrine hydrochloride present from the standard values obtained at the same time the sample is analyzed.

DISCUSSION AND RESULTS

The ion exchange resins used successfully under these conditions are Dowex 50-X-1, 2, 8, 12, and 16, Dowex 50-W-X-1, and Amberlite IR-120 analytical grade. With Dowex 50-X-1 in the sodium form, phenylephrine is also recovered quantitatively.

As much as 100 mg. phenylephrine hydrochloride can be held by the resin column and subsequently eluted and determined quantitatively (100 ± 2% recovery). In using these columns one must consider the presence of all other basic substances as well as salts which are present, as the resin may become so saturated that the phenylephrine is not retained during the water washes. It should be kept in mind that neutral salts such as sodium chloride are split by the resin so that an equivalent amount of hydrochloric acid is released. If the wash water contained over 0.1 *M* acid, the phenylephrine would be eluted. With the various formulations analyzed no such interference has been noted.

Phenylephrine is taken and held by the resin from aqueous solutions containing as much as 0.1 *M* hydrochloric acid so the original sample or extract can be this acidic without affecting the recovery.

Once the phenylephrine is held by the resin any amount of water may be passed through the column. The amount of washing depends on the amount and kinds of interfering substances removed by this step.

Quantitative acid elution requires a minimum of 0.2 *M* hydrochloric acid. The maximum permissible molarity depends upon the elution characteristics of other basic substances which may be held by the column. Acid above 1 *M* elutes thenyldiamine and interferes with the azo dye method. Experience has shown that different lots of nominally the same resin require somewhat different acid strength to get recoveries of 100 ± 2%. For most purposes, 0.5 *M* acid represents a good compromise, but it is advisable to check this point on each new lot of resin purchased.

Dowex resins show some shrinkage during the acid elution. Thus the volume of water freed from a given resin during the elution is variable. It follows that the acid molarity is variable, and therefore it is important to neutralize each eluate carefully before proceeding with color development.

The colorimetric methods are well known and have been modified only slightly, to fit the needs of the procedure presented. Only one point merits particular emphasis, namely that the azo coupling reaction must be run at about pH 9 (borax) to get good color production.

In the technique described for the Millon method, the nitric acid resin eluate is colorless as it comes off the column. If a catecholamine is also in solution, the eluate soon turns yellow. This color does not interfere with the assay. If a precipitate occurs during colorimetry, the presence of chloride ion should be suspected. However, if glassware is scrupulously clean and the directions are carefully followed, the solutions will remain clear and the color stable for at least three days.

The recommended procedure was developed to handle the analysis of a tablet of the following composition:

	Per Tablet, mg.
N-Acetyl-*p*-aminophenol (APAP)	150
Phenylephrine hydrochloride	5
Thenyldiamine hydrochloride	7.5
Caffeine	15

Both thenyldiamine and APAP interfere with the phenylephrine determination, thus preliminary physical separation is required. All the intact APAP and over 99% of the caffeine pass through the column during the washing step while the phenylephrine and thenyldiamine remain fixed to the column. On eluting with 0.5 *M* hydrochloric acid only the phenylephrine is removed. Of course, new interferences may appear as the result of decomposition during stability studies. Thus APAP might conceivably hydrolyze to *p*-aminophenol (PAP), a serious interference. For a purely hypothetical case, if 1% of the APAP were hydrolyzed, the final colorimetric solution would contain 4.3 mcg. PAP which would read as 0.9 mcg. phenylephrine hydrochloride, and would thus result in a falsely high value for phenylephrine.

As a matter of fact, during stability studies at 40° for a year, little change was noted in phenylephrine values for the formulation quoted.

A large number of other complex formulations, including syrups and elixirs, as well as tablets, have been assayed successfully by the recommended procedure. Other substances in these formulations included acetophenetidin, dextromethorphan hydrobromide, pyrilamine maleate, aspirin, phenolphthalein, various common pharmaceutical dyes, ascorbic acid, codeine phosphate, chlormezanone, chlorpheniramine maleate, meperidine, potassium guaiacolsulfonate, potassium iodide, ammonium chloride, benzocaine, bismuth subgallate, tannic acid, boric acid, various suppository bases, and catecholamines. As already indicated, only when catecholamines are present is it useful to abandon the azo colorimetric procedure and resort to Millon's reagent.

SUMMARY

Working details have been presented for the isolation (via a cationic exchange resin) and colorimetric determination of intact phenylephrine in various pharmaceutical preparations. The combination ion exchange-colorimetric procedures measure intact phenylephrine only: the ion exchange involves the basic nitrogen side chain while the colorimetric methods (coupling with diazotized *p*-nitroaniline or reaction with Millon's reagent) depend upon the presence of the unchanged phenolic group.

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Relative Hydrolytic Rates of Certain Alkyl (b) *dl*-α-(2-Piperidyl)-phenylacetates

By PHILIP S. PORTOGHESE and LOUIS MALSPEIS

A series of alkyl esters of (b) *dl*-α-(2-piperidyl)-phenylacetic acid have been prepared and the kinetics of the hydroxyl ion-catalyzed hydrolysis at 80° and the hydronium ion-catalyzed hydrolysis at 95° of six of these esters were studied. In the base-catalyzed reaction, the following order of reactivity was found: 2-chloroethyl > 2-methoxyethyl > methyl > ethyl > *n*-propyl > *n*-butyl. The apparent free energy reaction constant for the base-catalyzed hydrolysis of this series was determined to be +1.372 and, from the substituent constants reported in the literature, the relative rates of the isopropyl, isobutyl, and *sec*-butyl esters were calculated. The relative central stimulating activities of this series of esters reveals a decrease in activity with an increase of alkyl chain length.

THE DIASTEREOISOMER designated as the (b) racemate of methyl α-(2-piperidyl)-phenylacetate[1] is a mild central nervous system stimulant without significant sympathomimetic and cardiovascular effects (1). In a previous paper (2) the kinetic parameters of the hydrolysis of this ester were reported. It was shown that the estimated isocatalytic point at 25° is 3.48. This comparatively low value of the isocatalytic point was attributed to the proximity effect of the cationic center on the carbomethoxy group. Inasmuch as this drug is utilized in parenteral solutions whose pH is greater than 3.48, the principal degradative process in these solutions results from hydroxyl ion catalysis.

The inhibition of the rates of hydrolysis of certain aromatic esters in solution by the formation of molecular complexes has been demonstrated by Higuchi, *et al.* (3–7). Effective complexing occurs with compounds having polar groups spatially oriented to interact with groups on the substrate molecule (7). Accordingly, it was of interest to determine whether molecules with polar functional groups at intramolecular distances complementary to the quaternary nitrogen and the carbalkoxy group in methyl (b) *dl*-α-(2-piperidyl)-phenylacetate would retard the rate of hydrolysis of the ester. The additives selected on this basis were caffeine, N,N-dimethylacetamide, N,N′-dimethylurea, nicotinamide, glycine, and serine. In addition, the effects of polyvinylpyrrolidone (PVP) and polyethylene glycol 20M (PEG 20M) on the rate of hydrolysis of the methyl ester were investigated.

Minor modification of an organic medicinal which undergoes hydrolytic degradation in aqueous solution may afford a compound with comparable pharmacologic activity and enhanced stability. That central stimulating activity of methyl (b) *dl*-α-(2-piperidyl)-phenylacetate is associated with the β-phenylethylamine moiety is indicated by structural analogy with 1-phenyl-2-aminopropane, 3-methyl-2-phenylmorpholine, and α-(2-piperidyl)-benzhydrol. Structural alteration of the alkyl group is considered *a priori* likely to influence the intensity rather than the type of pharmacologic activity. Moreover, esters of higher alcohols are expected to be more stable to both specific acid- and specific base-catalyzed hydrolysis. The present study was undertaken to compare the central stimulating activities of a series of esters of (b) *dl*-α-(2-piperidyl)-phenylacetic acid and to determine the relative rates of the hydronium and hydroxyl ion-catalyzed hydrolysis of the pharmacologically active esters.

EXPERIMENTAL

Preparation of Esters of the (b) Racemate of α-(2-Piperidyl)-phenylacetic Acid Hydrochloride.—Dry hydrogen chloride gas was passed through a solution of the (b) *dl*-α-(2-piperidyl)-phenylacetic acid (8) in a fourfold molar excess of the alcohol. Upon saturation, the resulting clear solution was heated on a water bath for two to three hours and then evaporated to dryness. For the esters of cyclohexanol and benzyl alcohol, the ester hydrochloride was precipitated from the reaction mixture by the addition of ether. The crude ester hydrochloride was washed with ether, dried, dissolved in water, and the solution was made alkaline with aqueous sodium hydroxide. Ether was added, the organic layer was separated, and the aqueous layer was extracted with ether. The combined extracts were dried over anhydrous calcium sulfate and hydrogen chloride gas was passed through the solution. The (b) racemate of the ester hydrochloride was filtered off, washed with ether, and recrystal-

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[1] Ritalin is the registered trademark of Ciba Pharmaceutical Products, Inc., Summit, N. J.

lized from a suitable solvent. The compounds prepared by this method are listed in Table I.

Analytical Procedures.—The iron(III) hydroxamic-acid colorimetric method previously employed (2) was used to follow the specific base-catalyzed rates of hydrolysis of the methyl ester in aqueous buffers and in the presence of potential complexing agents. It was found that none of the additives altered the absorption characteristics of the ferric hydroxamic-acid complex in the 475–575 mμ region and therefore, the rate of disappearance of the ester was followed by measuring the decrease in the intensity of the colored complex at 530 mμ. Two useful though minor modifications in the colorimetric method were introduced. It was noted that better reproducible results were obtained when the time allowed for hydroxamate formation was extended from fifteeen to thirty minutes. In addition, following the formation of the colored complex 95% ethanol in lieu of distilled water was used to dilute the mixture to volume. This latter procedure served the dual purpose of preventing the accumulation of gas bubbles on the walls of the cuvettes as well as effectively dissolving any phthalic acid which crystallized following acidification of the solution when phthalate buffer was utilized in the kinetic study.

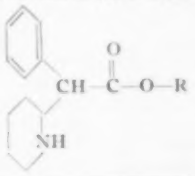
The applicability of the colorimetric procedure to the estimation of the esters listed in Table I was investigated. Into a 10-ml. volumetric flask containing 1.0 ml. of 1.0 *M* hydroxylamine hydrochloride was pipetted 2.0 ml. of 0.0056 *M* ester hydrochloride, followed by 1.0 ml. of 2.0 *M* sodium hydroxide in 95% ethanol. The flask was heated in a water bath at 70° for forty minutes, withdrawn from the bath, the solution acidified with 1.0 ml. of 2.0 *M* hydrochloric acid, and assayed in the usual way. It is evident from the following results that the rate of hydroxamate formation relative to hydrolysis is substantially lower with higher alkyl esters than with the methyl ester.

Ester	Absorbance at 530 mμ	Absorbance Relative to the Methyl Ester, %
Methyl	0.364	100
Ethyl	0.206	56
n-Propyl	0.150	41
n-Butyl	0.076	21
i-Butyl	0.078	22
n-Amyl	0.026	7

In order to increase the rate and extent of hydroxamate formation, the concentrations of the hydroxylamine and sodium hydroxide were increased, however, no increase in the absorbance was observed. Esters of secondary alcohols proved to be far less reactive than the esters of primary alcohols cited above; for example, application of the procedure to the isopropyl and *sec*-butyl esters yielded extremely little colored complex. It was of interest to note that the 2-methoxyethyl ester afforded an absorbance comparable to that of the methyl ester. Apparently, electrophilic substituents facilitate formation of the hydroxamate, undoubtedly by decreasing the electron density on the carbonyl-carbon so as to render it more susceptible to nucleophilic attack.

Inasmuch as the iron (III) hydroxamic-acid colorimetric method was demonstrated to be unsuitable for the analysis of most of the esters in Table I, the applicability of the previously described Extraction Method (2) was examined. Essentially, this is a direct spectrophotometric assay requiring the separation of the ester from the acid. Separation was achieved by making the hydrolysis mixture alkaline with sodium hydroxide solution thereby converting the acid to the water-soluble sodium salt and the ester hydrochloride to the free base. The free base was extracted from the aqueous phase with cyclohexane, and the absorbance of an aliquot of the cyclohexane extract was determined at 259 mμ. The absorbance of the solutions was measured in the Beckman model DU spectrophotometer using matched quartz cells.

TABLE I.—HYDROCHLORIDES OF ALKYL (b) *dl*-α-(2-PIPERIDYL)-PHENYLACETATES



R	Yield, %[a]	M.p., °C.[b]	Formula	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found	Nitrogen, % Calcd.	Nitrogen, % Found
Ethyl	84.2	210–212[d]	$C_{15}H_{22}ClNO_2$	63.48	63.79	7.81	7.90	4.94	5.02
n-Propyl	83.5	209–210[d]	$C_{16}H_{24}ClNO_2$	64.52	64.73	8.12	8.27	4.70	4.69
i-Propyl	44.9	227–228[e]	$C_{16}H_{24}ClNO_2$	64.52	64.26	8.12	8.11	4.70	4.75
n-Butyl	48.0	165–166[c,d]	$C_{17}H_{26}ClNO_2$	65.47	65.65	8.40	8.48	4.49	4.57
i-Butyl	61.5	213–214[d]	$C_{17}H_{26}ClNO_2$	65.47	65.41	8.40	8.54	4.49	4.45
sec-Butyl	70.5	208–209[d]	$C_{17}H_{26}ClNO_2$	65.47	65.61	8.40	8.62	4.49	4.51
n-Amyl	61.4	174–177[d]	$C_{18}H_{28}ClNO_2$	66.34	65.93	8.66	8.78	4.30	4.15
Cyclopentyl	70.7	229–230[d]	$C_{18}H_{26}ClNO_2$	66.75	65.92	8.09	8.20	4.33	4.26
Cyclohexyl	75.3	197–198[d]	$C_{19}H_{28}ClNO_2$	67.54	67.74	8.35	8.55	4.15	4.12
Benzyl	56.4	199–200[d]	$C_{20}H_{24}ClNO_2$	69.45	69.56	6.99	7.16	4.05	4.11
2-Methoxyethyl	88.6	170–171[d]	$C_{16}H_{24}ClNO_3$	61.24	61.57	7.71	7.75	4.46	4.52
2-Chloroethyl	71.0	169–170[f]	$C_{15}H_{21}Cl_2NO_2$	56.61	56.63	6.65	6.67	4.40	4.30

[a] Recrystallized yield. [b] The uncorrected values reported were determined by immersing the capillary tube into a dibutyl phthalate bath preheated to within approximately 10° of the melting point and subsequently heated at the rate of 1° per twenty seconds. [c] R. Rometsch (1), m. p. 165°. [d] Recrystallized from isopropanol. [e] Recrystallized from ethanol. [f] Recrystallized from isopropanol-ether.

Using a Cary model 11 recording spectrophotometer it was established that (b) *dl*-α-(2-piperidyl)-phenylacetic acid and its aliphatic esters (Table I) in cyclohexane show identical ultraviolet absorption spectra. The spectrum of the methyl ester is recorded in the previous paper (2). Each ester was shown to follow a Beer-type relationship over the concentration range used. Those esters whose alkyl moieties are larger than ethyl exhibited decreased solubility in cyclohexane. To facilitate extraction cyclohexane-ether (7:3 by volume) was used in place of cyclohexane.

In order to determine how completely each ester was extracted, dilutions were made of a cyclohexane-ether solution of the ester and the absorbances of these dilutions were then compared with those obtained when aqueous solutions containing identical ester concentration were extracted with cyclohexane-ether, as described in the analytical method. In each case, the two absorbance-concentration curves obtained in this way were virtually coincident. Thus, the partition ratio for each ester was so large that for all practical purposes the residual concentration of ester in the aqueous phase was negligible.

The pseudo first-order rate constants for the hydrolysis of the methyl ester at 80°, pH 5.98, and ionic strength 0.85 were determined by the colorimetric assay method to be 3.223×10^{-4} min.$^{-1}$ and by the extraction procedure to be 3.179×10^{-4} min.$^{-1}$. Hence, the correlation between the two assay procedures is excellent, the values obtained being within the usual estimated reproducibility of first-order constants.

The extraction method of analysis was used to obtain the pseudo first-order rate constants of the alkyl (b) *dl*-α-(2-piperidyl)-phenylacetates.

Buffer Solutions.—Siegel, *et al.* (2), established the absence of general acid or general base catalysis with the components of phthalate buffer. Unfortunately, at pH 6 the capacity of this buffer is somewhat limited and relatively high concentrations of the buffer components are therefore required. The buffer employed for the determination of the reaction rates of the hydroxyl ion-catalyzed ester hydrolysis was prepared 0.300 *M* in potassium biphthalate and 0.272 *M* in sodium hydroxide. The ionic strength of the resultant buffer was calculated to be 0.85 and the pH was found to be 5.82 at 23.0° and 5.98 at 80.0°.

The medium for the hydronium ion-catalyzed hydrolyses was 0.100 *M* hydrochloric acid containing a quantity of sodium chloride sufficient to adjust the final ionic strength to 0.85. The pH of this solution was 0.97 at 22.0° and 1.14 at 95.0°.

It was established that the buffer capacity was adequate to maintain constant hydronium ion concentration throughout the kinetic runs. The pH was measured before, during, and after each run.

At room temperature pH was measured with both the Beckman model G pH meter and the Beckman model H-2 pH meter, and the latter instrument was used for determinations at elevated temperatures. The meter was standardized at 80.0 and 95.0° with Beckman standard buffer, pH 7.00, using the temperature corrections cited on the container and with phthalate buffer, pH 4.00, employing the temperature corrections cited by Britton (9).

Kinetic Runs.—A solution of the recrystallized ester hydrochloride (5.6×10^{-3} *M*) in the appropriate buffer was injected into U. S. P. XV type I ampuls and the ampuls were sealed. The ampuls were immersed in a liquid constant temperature bath, maintained to within ±0.05°; and the solution was brought to thermal equilibrium. At various intervals the ampuls were withdrawn and the reaction quenched by plunging the ampul into an ice-water bath. The samples were analyzed for unreacted ester using the extraction method of Siegel, *et al.* (2). The absorbances were measured at 259 mμ in a Beckman model DU spectrophotometer.

Characteristic pseudo first-order plots for the rate of disappearance of the reactant were obtained for at least one-half time in all experiments. In a typical experiment, 10 samples were taken during this time interval. The specific first-order rate constants were estimated directly from the plots.

RESULTS AND DISCUSSION

Effect of Additives on the Specific Base-Catalyzed Hydrolysis of Methyl (b) *dl*-α-(2-Piperidyl)-phenylacetate.—Siegel, *et al.* (2), have reported that the approximate pKa of the methyl ester is 8.8 so that in a solution whose pH is 7 and below, it is the cationic ester which is subject to hydrolysis. Inasmuch as the isocatalytic point of the hydrolysis is 2.86 at 80.0°, catalysis at pH 5 and pH 7 is exclusively due to hydroxyl ion. Parenteral preparations containing this compound are buffered at a pH greater than the isocatalytic point so that, in these preparations, the degradation is predominantly specific base-catalyzed hydrolysis of the protonated ester. In order to evaluate the effects of the additives on the hydroxyl ion-catalyzed hydrolysis of the methyl ester, the kinetics of the reaction was followed in the absence and presence of the additive. Identical hydrolytic conditions were achieved by simultaneously immersing ampuls containing solutions buffered at pH 5 or 7 of the methyl ester (5.55×10^{-3} *M*) in the absence and presence of the additive into the constant temperature bath and withdrawing the ampuls at specified intervals. The "apparent" pH of each solution was measured at the elevated temperature. The "apparent" second-order rate constants were evaluated as described by Siegel, *et al.* (2). It is necessary to compare "apparent" second-order rate constants rather than pseudo first-order rate constants to accomodate for very minor changes in the hydroxyl ion concentration occurring in the presence of certain additives.

Table II summarizes the effects of the additives on the rate. Under the conditions described, glycine, serine, and PEG 20M were the only compounds of those investigated which retarded the hydrolysis rate to a small extent. The magnitude of the effects observed with glycine and serine is, within the experimental error, comparable to the negative salt effect calculated (2) from $\log k = \log k_s^s - 0.307 \sqrt{\mu}$.

In a study of the rate of hydrolysis of benzocaine in the presence of nonionic surfactants, Riegelman (10) observed that the half-life of this ester (1 mg. %) was extended almost fourfold through the addition of 3.3% cetyl alcohol polyoxyethylene-14

TABLE II.—RELATIVE SECOND-ORDER RATE CONSTANTS OF THE HYDROLYSIS OF METHYL (b) *dl*-α-(2-PIPERIDYL)-PHENYLACETATE IN THE PRESENCE AND ABSENCE OF ADDITIVES

Compound	Concn.[a]	Apparent pH[b]	Temp., °C.	Buffer[c]	k_c/k_e[d]
Caffeine	5	7.00	50.0	TEA	1
Caffeine	20	7.00	50.0	TEA	1
N,N-Dimethylacetamide	10	7.00	50.0	TEA	1
N,N-Dimethylacetamide	20	7.00	50.0	TEA	1
Glycine	10	7.00	50.0	TEA	0.93
Serine	10	7.00	50.0	TEA	0.90
N,N'-Dimethylurea	20	7.00	50.0	TEA	1
Nicotinamide	10	5.00	80.0	Phthalate	1
Polyvinylpyrrolidone	5%	5.00	80.0	Phthalate	1
Polyethyleneglycol 20M	5%	5.00	80.0	Phthalate	0.85

[a] Concentration expressed as a multiple of the molar ester concentration except in instances where the additive is a polymer and concentration is expressed as % w/v.
[b] pH values measured with glass calomel electrodes at the temperatures cited.
[c] TEA buffer is 0.11 *M* in triethanolamine and 0.09 *M* in triethanolammonium chloride. Phthalate buffer is 0.105 *M* in potassium hydrogen phthalate and 0.095 *M* in potassium sodium phthalate.
[d] k_c is the second-order rate constant of the hydroxyl ion-catalyzed hydrolysis of the ester in the presence of the additive. k_e is the second-order rate constant in the absence of the additive. A ratio designated as 1 indicates that k_c is within ±3% of k_e.

TABLE III. FIRST-ORDER RATE CONSTANTS AND RELATIVE RATES OF THE HYDROLYSIS OF ALKYL (b) *dl*-α-(2-PIPERIDYL)-PHENYLACETATES

Ester	Hydroxyl Ion-Catalysis $k \times 10^3$, min.$^{-1}$[a]	$(k/k_0)_B$[b]	Hydronium Ion-Catalysis $k \times 10^4$, min.$^{-1}$[c]	$(k/k_0)_A$[b]
Methyl	3.179	1.000	8.781	1.000
Ethyl	1.628	0.512	7.054	0.803
n-Propyl	1.522	0.429	6.860	0.781
n-Butyl	1.283	0.404	6.424	0.732
2-Methoxyethyl	3.940	1.239	7.007	0.798
2-Chloroethyl	9.249	2.905	12.709	1.447
i-Butyl	1.069[d]	0.336[d]	....	....
i-Propyl	0.259[d]	0.082[d]	....	....
sec-Butyl	0.142[d]	0.045[d]	....	....

[a] Temperature 80.0°; phthalate buffer, 0.028 *M* potassium hydrogen phthalate and 0.272 *M* potassium sodium phthalate; ionic strength 0.85; pH 5.98 at 80.0°.
[b] k_0 = methyl ester = 1.
[c] Temperature 95.0°; hydrochloric acid 0.100 *M* adjusted to ionic strength 0.85 with sodium chloride; pH 1.14 at 95.0°.
[d] Calculated from σ values listed in Table IV and ρ = +1.372.

ether, and he noted that the ester is hydrolyzed within the micelle and in the aqueous phase. It appears that the rate of hydrolysis of this aromatic ester is decreased to a greater extent by micelle inclusion than is the rate of hydrolysis of the aliphatic ester in the present study.

Relative Rates of the Hydrolysis of Alkyl (b) *dl*-α-(2-Piperidyl)-phenylacetates—The observed rates of hydrolysis of six pharmacologically active esters of the (b) racemate of *dl*-α-(2-piperidyl)-phenylacetic acid at 80.0° at pH 5.98, and at 95.0° at pH 1.14 are recorded in Table III.

The specific acid-catalyzed reaction was carried out with approximately a twentyfold excess of hydronium ion over ester and the specific base-catalyzed reaction was carried out in phthalate buffer. All solutions were adjusted to an ionic strength of 0.85 with sodium chloride. In each case a straight line relationship was obtained in a plot of log residual ester concentration against time, demonstrating pseudo first-order kinetics. Duplicate experiments indicated that the precision of the first order experiments is within ±4%. A typical pseudo first-order plot is shown in Fig. 1.

It was observed with the esters of secondary alcohols that at pH 6, a pale straw-yellow color gradually developed during the reaction. An ultraviolet absorption spectrum of a solution of the *sec*-butyl ester which had been hydrolyzed, based upon

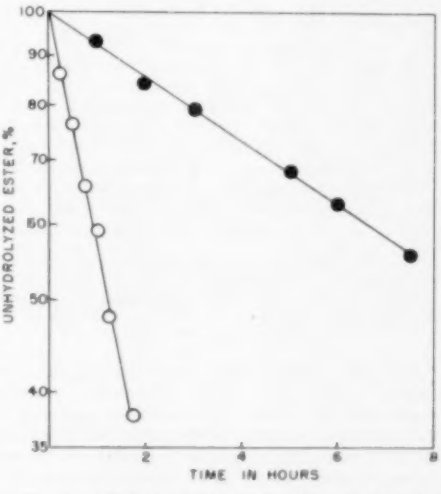


Fig. 1.—Typical pseudo first-order rate plots for the hydrolysis of alkyl (b) *dl*-α-(2-piperidyl)-phenylacetates: ●, hydronium ion-catalyzed hydrolysis of the 2-methoxyethyl ester at 95.0°, pH 1.14; ○, hydroxyl ion-catalyzed hydrolysis of the 2-methoxyethyl ester at 80.0°, pH 5.98.

the initial slope of a pseudo first-order plot, for a period greater than four half-times, showed a very broad absorption peak at 293 mμ which extended into the 259 mμ region (Fig. 2). The apparent deviation from a typical pseudo first-order plot observed on saponification of the *sec*-butyl ester (Fig. 3) can be attributed to a reaction occurring consecutive to or parallel with the hydrolysis. Although Fig. 3 suggests that the deviation from first-order kinetics occurs only on the appearance of the yellow color, it is evident from a plot showing the increase in the absorbance at 293 mμ with time as the hydrolysis reaction proceeds (Fig. 4) that the product of the second reaction is contributing to the absorbance at 259 mμ during the early stage of the hydrolysis.

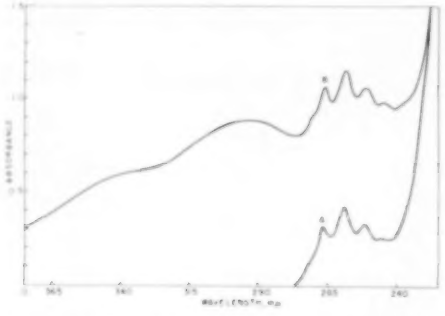


Fig. 2.—Ultraviolet spectra: *A*, *sec*-butyl (b) *dl*-α-(2-piperidyl)-phenylacetate, 3.2×10^{-4} *M* in water; *B*, prolonged hydrolysis of the *sec*-butyl ester at 95.0°, pH 6.92.

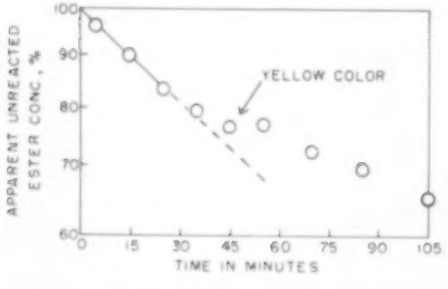


Fig. 3.—The observed pseudo first-order plot of the hydrolysis of *sec*-butyl (b) *dl*-α-(2-piperidyl)-phenylacetate at 95.0°, pH 6.92, determined by the extraction procedure.

The yellow color also was noted upon heating of an aqueous solution of (b) *dl*-α-(2-piperidyl)-phenylacetic acid at 95.0°. Moreover, the increase in the absorbance at 293 mμ with time paralleled the increase observed during the saponification of the *sec*-butyl ester. These observations indicate that the reaction yielding the yellow-colored product is consecutive to the hydrolysis through the intermediate carboxylic acid. Extraction of the degradation product into cyclohexane from a strongly basic solution indicates the absence of a carboxyl group. Furthermore, it was found that little

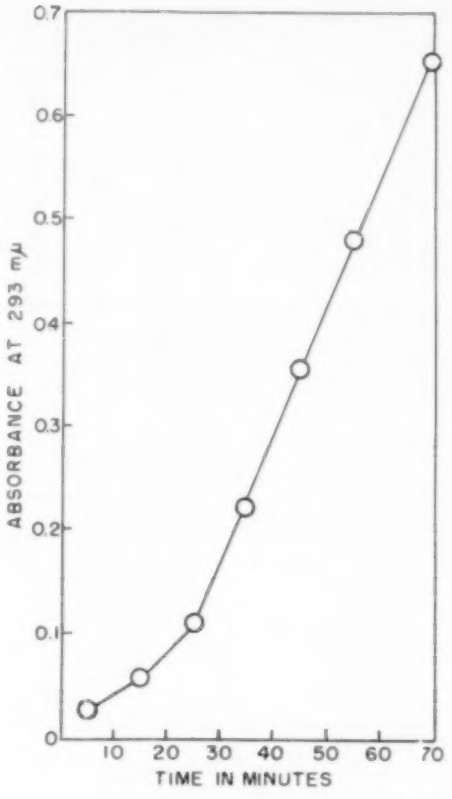


Fig. 4.—Rate of the decarboxylation of the zwitterionic acid resulting from the hydrolysis of *sec*-butyl (b) *dl*-α-(2-piperidyl)-phenylacetate at 95.0°, pH 6.92.

degradation occurs on heating a strongly basic or acidic solution of the carboxylic acid. The relative stability of this 2-piperidylacetic acid in acidic or basic solution and its instability in neutral solution are in agreement with the observations of Doering and Pasternak (11) that the decarboxylation of neutral methylethyl-α-pyridylacetic acid takes place much more readily than either the pyridinium salt or the carboxylate ion. Therefore, it is suggested that following ester hydrolysis the zwitterionic 2-piperidylacetic acid is decarboxylated and that the observed yellow color is due to the small amount of enamine in equilibrium with the resulting 2-benzylpiperidine. It is of interest to note that the decarboxylation appears to follow overall zero order kinetics (Fig. 4).

No yellow color was seen in the solutions of esters of primary alcohols during the kinetic runs. Under the milder conditions required for the hydrolysis of these compounds no appreciable amount of decarboxylation appears to occur until the reaction has proceeded through several half-times.

The rates of hydrolysis of the alkyl esters relative to the methyl ester are expressed in Table III as k/k_0, where k is the rate constant of the alkyl ester and k_0 is the rate constant of the methyl ester. In

the hydroxyl ion-catalyzed hydrolysis the expected decrease in the reaction velocity in the series methyl, ethyl, *n*-propyl, *n*-butyl parallels the increasing positive inductive effect. The increase in the base-catalyzed hydrolytic rates of the 2-methoxyethyl and 2-chloroethyl esters relative to the ethyl ester is qualitatively in accord with the electron withdrawing properties of the methoxy and chloro substituent groups (12). These results agree well with the rates of alkaline hydrolysis of alkyl esters of an aliphatic acid (13–15) and illustrate that in this reaction the velocity is predominantly influenced by the electron density on the carbonyl-carbon in accord with Bender's mechanisms of ester hydrolysis (16).

Inasmuch as the analytical procedure employed in the present study does not permit the experimental determination of the hydroxyl ion-catalyzed hydrolysis rates of the relatively stable esters (Fig. 3), these rates were predicted by means of the Hammett equation, $\log k/k_0 = \rho\sigma$ (13). In order to obtain the free energy reaction constant, ρ, σ values for the standard reaction series, the hydrolysis of alkyl acetates, for the same solvent and at the same temperature must be known; however, this data is not available. Furthermore, Taft (17) has indicated that σ values estimated from reactions in aqueous organic solvents do not apply well to reactions carried out in pure water, thereby precluding the use of most values cited in the literature. The influence of the solvent system on the energy of activation for the alkaline hydrolysis of esters has been discussed (2). Nevertheless, an apparent ρ may be obtained by the use of σ values for the base-catalyzed hydrolysis of alkyl acetates in water at 20.0° (Table IV). The correlation of the rates of

TABLE IV.—SUBSTITUENT CONSTANTS, σ, FOR THE SPECIFIC BASE-CATALYZED HYDROLYSIS OF ALKYL ACETATES

Alkyl	k'/k'_0[a]	σ
2-Methoxyethyl	1.119[b]	+0.049
Methyl	1.000[c]	0.000
Ethyl	0.583[c]	−0.234
n-Propyl	0.540[c,d]	−0.268
n-Butyl	0.501[c,d]	−0.300
i-Propyl	0.161[d]	−0.793
i-Butyl	0.452[c,d]	−0.345
sec-Butyl	0.104[c,d]	−0.983

[a] k'_0 = methyl acetate = 1, T = 20.0°.
[b] 16.67% (vol.) dioxane-water, T = 25.0°. Salmi, E. J., and Leimu, R., *Suomen Kemistilehti*, **20B**, 43(1947); through *Chem Abstr.*, **42**, 4031(1948).
[c] Smith, L., and Olsson, H., *Z. Physik. Chem.*, **118**, 99 (1925).
[d] Olsson, H., *ibid.*, **118**, 107(1925).

the base-catalyzed hydrolysis of alkyl (b) *dl*-α-(2-piperidyl)-phenylacetates at 80.0° with those of alkyl acetates at 20.0° by the Hammett equation is shown in Fig. 5. Using the least squares method (18) for determining the regression it is found that $\rho_{app.} = +1.372 \pm 0.018$. The correlation coefficient is 0.998. Compared with the free energy reaction constants of 0.820 for the hydrolysis of acetate esters in water at 25° and 1.000 for the hydrolysis of benzoate esters in 60% (vol.) aqueous dioxane at 25° (13), this higher "apparent" ρ value reflects the high collision factor contributing to the energy of activation in this reaction (2) and the contribution

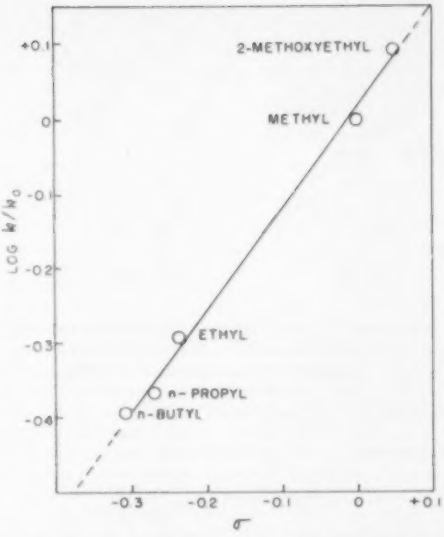


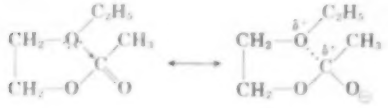
Fig. 5.—Correlation of log k/k_0 values for the specific base-catalyzed hydrolysis of alkyl esters of (b) *dl*-α-(2-piperidyl)-phenylacetic acid (phthalate buffer, pH 5.98; T = 80.0°; μ = 0.85) with σ values of alkyl acetates (T = 20.0°).

of steric factors in the acyl moiety to the relative free energy of activation (14). The first-order rate constants of the hydroxyl ion-catalyzed hydrolysis of the *i*-propyl, *i*-butyl, and *sec*-butyl esters were calculated from the σ values of the standard reaction series (Table IV) and are listed in Table III.

The relative rates of the hydronium ion-catalyzed hydrolysis of the methyl, ethyl, *n*-propyl, and *n*-butyl esters (Table III) are qualitatively similar though smaller in magnitude than those observed in the base-catalyzed reaction. Taft (19), assuming that the relative rates in acid-catalyzed ester hydrolysis are independent of polar effects, has defined $E_s \equiv \log (k/k_0)_A$ as "a near-quantitative measure of the total steric effect associated with a given substituent relative to the standard of comparison." The values of E_s at 25° calculated by Taft for CH_3, C_2H_5, n-C_3H_7, and n-C_4H_9 as the acyl component of aliphatic esters are 0.00, −0.07, −0.36, and −0.39, respectively, and the values of E_s at 95° for these substituents as alkyl components are 0.00, −0.09, −0.11, and −0.14, respectively. Thus, a similar order of steric interaction of these substituents is observed in both the alkyl and acyl groups.

The relative rates of the hydrolysis of the 2-chloroethyl and 2-methoxyethyl esters are particularly noteworthy. The ratios $(k/k_0)_B$ for the hydroxyl ion-catalyzed hydrolysis of the 2-chloroethyl and 2-methoxyethyl esters are +2.905 and +1.239, respectively. The magnitude of these relative rates is appreciably higher than indicated by the negative inductive effects of a chloro or a methoxy group in the beta-position. For the chloroethyl group as the acyl component, k/k_0 for basic hydrolysis is 1.135 (20). It is evident that there is a considerable difference in this case in the polar effect of the acyl and alkyl substituent.

Rabinovitch and Schramm (21) postulated that the higher energy of activation for the basic hydrolysis of 2-ethoxyethanol-1 acetate (15.4 Kcal.) compared with ethyl acetate or isopropyl acetate (11.7 Kcal.) is due to the formation of a quasi-five-membered ring in which "the ether-oxygen atom can partially donate its unpaired electrons to the carbon atom."



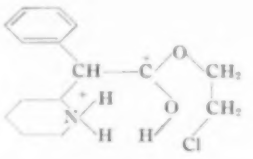
It is noted that the relative rates of basic hydrolysis of the 2-methoxyethyl to the ethyl ester, 2.42, found in the present investigation is consistent with the rate of hydrolysis of 2-ethoxyethanol-1 acetate relative to ethyl acetate at 25°, 2.41.

Although five-membered ring formation appears reasonable, it does not seem likely that the donation of electrons to the carbonyl carbon would result in increased electrophilicity of the carbon atom. Instead, it is speculated that the carbonyl-carbon becomes more electrophilic as a result of a proximity effect on the carbonyl-oxygen which can be exerted by the electronegative chlorine of the chloroethyl ester and by the oxygen of the methoxyethyl ester. The enhanced electrophilicity of the carbonyl-carbon as a consequence of these negative polarizability effects should be reflected in an increased relative reaction rate which is reduced somewhat by steric interference.

The relative rates, $(k/k^0)_A$, of the acid-catalyzed hydrolysis of the 2-chloroethyl and 2-methoxyethyl esters at 95.0° are 1.447 and 0.798. If it is assumed that polar effects are negligible in acid-catalyzed ester hydrolysis, the predominant influence on the relative rates is the steric effect. Accordingly, these relative rates are most unusual. In the case of the 2-chloroethyl ester, a bulky substituent compared with the methyl group, the reaction proceeds at a rate much faster than that of the methyl ester. Moreover, in the aliphatic series the steric effect of a chloro group is only slightly greater than that of a methoxy group as reflected by E_s values of −0.24 and −0.19 for the acyl substituents $ClCH_2$ and CH_3OCH_2, and −0.90 and −0.77 for the acyl substituents $ClCH_2CH_2$ and $CH_3OCH_2CH_2$ (20). In the present study the log $(k/k^0)_A$ values for the 2-chloroethyl ester, +0.16, differs from the value for the 2-methoxyethyl ester, −0.10. Finally, the comparable relative rates of the ethyl and the 2-methoxyethyl esters seems anomalous considering that the 2-methoxyethyl group is the bulkier substituent.

In acid-catalyzed ester hydrolysis, the rate-determining step is the attack of the dipolar water molecule on the carbonyl-carbon of the protonated ester (16). An increase in the electrophilic character of the carbonyl-carbon should facilitate the formation of the tetrahedral intermediate; however, it seems that inductive effects in the alkyl or acyl components cannot be transmitted to the carbonyl-carbon when the oxygen atoms are protonated. Consequently, steric factors predominate. On the other hand, through proximity effects, the electron density on the carbonyl-carbon may be altered (2) and it is plausible that the proximity effects discussed earlier are also operative in the acid-catalyzed reaction.

With the alkyl 2-piperidylacetates the reacting species is doubly protonated. The negative polarizability effect of the chlorine atom on the carbonyl-carbon in the cyclic conformation of the 2-chloroethyl ester should result in an increased rate of formation of the transition state complex. As before, the rate should be diminished by steric interference.



With the 2-methoxyethyl ester, the reacting species is triply protonated since the methoxy-oxygen is also protonated. In this species, there are two cationic centers in the neighborhood of the carbonyl-carbon and it seems likely that the protonated methoxy-oxygen will be largely repelled. In the favored conformation, the reaction should be greatly facilitated; whereas in most others, steric interference should result. The observed relative rate for the acid-catalyzed hydrolysis of the 2-methoxyethyl ester suggests that steric interference predominates.

Relative Central Stimulating Activities.—The relative central stimulating activities of the alkyl (b) *dl*-α-(2-piperidyl)-phenylacetates are presented in Table V. The bioassay technique utilized for assessing the values cited is that of Plummer, *et al.* (22).

Table V.—Relative Central Stimulating Activity of Alkyl (b) *dl*-α-(2-Piperidyl)-phenylacetates

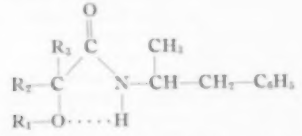
Alkyl	Central Stimulating Activity Relative to the Methyl Ester
Methyl	1.00
Ethyl	0.80
n-Propyl	0.20
i-Propyl	0.33
n-Butyl	0.13
i-Butyl	0.10
sec-Butyl	0.20
n-Amyl	<0.10
Cyclopentyl	<0.10[a]
Cyclohexyl	<0.10
Benzyl	<0.10
2-Methoxyethyl	0.20
2-Chloroethyl	0.13

[a] Activity is of an intermittent type rather than the sustained activity characteristic of the methyl ester.

The action of the central stimulants, which have as a common structural feature the 2-phenylisopropylamine group, has been attributed to monoamine oxidase inhibition in the brain, direct stimulation of the CNS, or to a combination of these effects (23). Those compounds which are able to reverse the CNS depression of reserpine, when administered

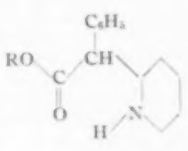
subsequent to the depressant, exert their analeptic action directly. Both *d*-α-methylphenethylamine and methyl (b) *dl*-α-(2-piperidyl)-phenylacetate elicit this type of reserpine reversal (24, 25) and, therefore, it is valid to compare their structures.

Shapiro and his co-workers (26) have compared the central stimulant activity of a series of amides of *d*-α-methylphenethylamine. They observed that with the exception of the formamide, the retention of excitant activity is associated with the α-oxy function on the acyl group and proposed that the unique structural feature common to the highly active compounds is the formation of a hydrogen bonded cyclic structure. For example, N-(*d*-α-methylphenethyl)-lactamide (R_1 = H, R_2 = CH_3, R_3 = H) is very highly active.



This contention was supported by the observation that high activity was found with compounds in which R_1 is a hydrogen atom or a substituent exerting a positive inductive effect, whereas the excitant activity was considerably diminished with those compounds in which R_1 was a group producing a reduction in the electron density on the adjacent oxygen. On the other hand, the compound in which there are two methyl groups on the α-carbon atom (R_1 = H, R_2 = CH_3, R_3 = CH_3) produces CNS depression rather than excitation although conceivably intramolecular hydrogen bonding can occur. Moreover, the compound in which there is a large alkyl group on the α-carbon (R_1 = H, R_2 = 3-heptyl, R_3 = H) also produces a depressant effect.

The relationship of the above hydrogen bonded structure to methyl (b) *dl*-α-(2-piperidyl)-phenylacetate (R = CH_3) was noted by Shapiro, *et al.* (26).



This compound is highly structurally specific; analeptic activity is associated with one enantiomer of the (b) diastereoisomer (1). The tabular summary of the comparative central stimulating activity of the alkyl esters of the 2-piperidylacetic acid indicates that only the methyl ester exhibits a high order of activity. The relative rates of the hydroxyl ion-catalyzed hydrolysis of the *n*-alkyl esters is a measure of the increasing positive inductive effect in this series. If intramolecular hydrogen bonding does occur in this molecule, it should be enhanced as the *n*-alkyl series is ascended. In addition, these *n*-alkyl esters would be expected to be potent CNS stimulants if the ability to form the cyclic structure were the significant structural parameter. It is evident that a decrease in activity occurs with an increase in the chain length or the size of the alkyl moiety. The comparable potency of the *n*-propyl and the 2-methoxyethyl esters indicates that analeptic activity is not related to the relative electron density at the carbonyl function and suggests that the decrease in activity relative to the methyl ester may be related to a steric factor or to decreased availability at the site of action.

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Investigation of the Cardiovascular Response of the Dog to 1-Phenyl-2-hydrazinopropane

By WILLIAM G. GROVES† and HOWARD J. JENKINS

Results of an attempt to determine the basis for the qualitative difference in blood pressure response of the human and the dog to 1-phenyl-2-hydrazinopropane are reported. This difference is not attributable to anesthesia or to mode of administration of the compound, nor is it involved with the recording position of the individual. A possible explanation for the difference, which is involved with action mechanism, is provided.

IT HAS BEEN stated by Horita that 1-phenyl-2-hydrazinopropane, also referred to as α-methyl phenethylhydrazine,[1] is a potent and long-lasting monoamine oxidase inhibitor (1). Eltherington and Horita have reported that it produced a pressor response in the anesthetized dog (2). Certain investigators have shown it to be 10 times as active as iproniazid in eliciting a rise in brain levels of norepinephrine and serotonin (3). Work by Brodie, Spector, Kuntzman, and Shore in 1958 has indicated, however, that it blocks norepinephrine synthesis *in vitro* by inhibiting the formation of dihydroxyphenylethylamine, a precursor of norepinephrine (4). Accordingly, studies in man have indicated that it has hypotensive activity (5). In individuals with elevated blood pressures, it has demonstrated a potent antihypertensive effect, the onset of which is rapid and the duration of which is considerable. In these studies, there occurred no decrease in supine blood pressure, but a substantial fall in standing pressure was notable. This finding is in sharp contrast to the pressor activity finding reported in dogs.

EXPERIMENTAL

Methods.—In an attempt to resolve the differences in blood pressure effects obtained with intravenous doses in anesthetized dogs and those obtained with oral doses in conscious humans, most of the recording of this work was accomplished in trained, conscious dogs to whom doses were administered both intravenously and orally. Thus, it could be shown whether the anesthesia was responsible for the differences, and also whether the route of administration had any bearing in the matter.

In order to carry out the work in conscious animals, it was necessary to train dogs so that femoral arterial pressure and respiratory tracings could be made with the aid of a local anesthetic only. Recordings were obtained with the Sanborn Twin-Viso recorder by means of Sanborn pressure transducers 467A–467B and Sanborn strain gauge amplifiers. Blood pressures were recorded in surgically intact, conscious dogs by making a puncture of the femoral artery with a 21-gauge needle. The puncture area was previously infiltrated with 2% procaine hydrochloride. Respiration was recorded by means of a bellows-type pneumograph which was connected to one of the pressure transducers. Throughout the course of the oral dose schedule, blood pressure recordings were made in both the supine and standing positions in the trained dog.

Results.—Prior to administering 1-phenyl-2-hydrazinopropane to conscious dogs, several doses were given to pentobarbital-anesthetized animals in an attempt to reproduce the results of cardiovascular studies already reported in the literature. Data thus obtained were qualitatively but not quantitatively like those reported. Whereas a 35% increase in mean blood pressure had been reported for the 1 to 2 mg. per Kg. dose range, results of this duplicate testing showed an increase in mean blood pressure of up to 147% over the same dose range.[2]

Further observations in anesthetized dogs indicated that a cardiac rate decrease occurs during the rise to peak pressure. This rate decrease did not appear to be necessarily reflex-ordered, since it always occurred at the beginning of the blood pressure rise rather than at or after the peak was reached. The peak blood pressure was reached, in every instance, within one and one-half to three minutes following the compound. Administered to anesthetized dogs, it increased respiratory rate if this rate was low, and depressed it if it was high.

In conscious dogs, the blood pressure effects are qualitatively similar to those in anesthetized animals. Also, in conscious animals, just as in anesthetized, there was a drop in cardiac rate during the blood pressure rise. There did not appear to be any significant difference in the duration of hypertensive action of this agent in the conscious and in the anesthetized dog. Respiration was generally decreased during the peak blood pressure rise, but this effect was of short duration only. Dogs which received more than one dose in a given run showed evidence of a tachyphylactic response with respect to blood pressure increase.

Following the higher doses of the drug in conscious dogs, a very noticeable sedating effect was

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[1] Supplied by Lakeside Laboratories as JB-516 and marketed as Catron.

[2] Mean blood pressure calculated by adding one-half of the pulse pressure to the diastolic pressure.

observed which had a duration of four to five minutes.

The most noticeable side effects from this drug included salivation, tachycardia, emesis, miosis, and skipped beats and other cardiac arrhythmias. In two dogs severe respiratory depression, almost continuous diarrhea, and prostration were observed.

Epinephrine hydrochloride, 0.1 ml. of 1:1000 solution, given intravenously following a dose of this amphetamine analogue produced a pressor response unlike that produced by an equivalent dose in an untreated animal. The pressure increase was normal in magnitude, but of greater duration than normal. There was no depressor response to the vasodilator component of epinephrine.

Because no significant differences in the responses of anesthetized and conscious dogs were observed following the drug (see Table I), it was deemed advisable to carry out an oral dosage schedule. One of the dogs which had been trained for conscious blood pressure determinations was selected for the work. Doses of 1 mg. per Kg. were given daily to the test animal over a fourteen-day period. Since according to clinical reports only standing blood pressure is lowered by this agent, both supine and standing blood pressures were recorded in the dog. This was done even though standing blood pressure effects are not equivalent in the dog and in man.

Blood pressure tracings were being taken prior to the drug, five hours following the first dose, and on the fourth, seventh, and fourteenth days of the schedule. Instead of a decrease in mean standing blood pressure, as reported in hypertensive humans, a definite increase was noted as early as five hours following the first dose. Both standing and supine pressures exhibited this increase, and both likewise reached a peak on the fourth day. A gradual decline then followed, so that on the fourteenth day supine blood pressure was back to normal and standing blood pressure was 27% above control levels (see Table II). Cardiac rate reached a peak on the seventh day (both standing and supine readings) while standing pulse pressure was greatest five hours after the first dose and supine pulse pressure was highest on the fourth day.

No prominent side effects were observed during the oral dose study, with the exception of a loose stool from the second to the fifth day and a sluggishness and a desire to lie down during the latter stages of the study.

Table I.—Blood Pressure Effects of 1-Phenyl-2-hydrazinopropane

Dose, mg./Kg.	Average % Rise, Mean Blood Pressure Anesthetized	Conscious
0.25	25	..
1.00	47	45
1.67	..	50
2.00	107	89
3.00	..	84
5.00	..	85

Table II.—Oral Dose Study: 1-Phenyl-2-hydrazinopropane

Day	Mean Blood Pressure, % of Control Supine	Standing	Cardiac Rate Supine	Standing	Pulse Pressure Supine	Standing
0	100	100	66	78	40	40
1	108	140	70	66	80	92
4	126	148	90	88	98	84
7	115	129	94	89	68	75
14	101	127	78	69	62	69

DISCUSSION

From the results of the preceding work, it may be concluded that neither anesthesia nor route of administration is responsible for the differences in blood pressure effects in conscious humans and those occurring early in anesthetized dogs.

Although the cardiac rate decrease in response to the drug did not appear to be reflex in nature, it is recognized that further checking of this point in the atropinized dog should be done.

In general, clinical results obtained with this amphetamine analogue have suggested the possibility that a strong depressor response is obtained only in the hypertensive individual.

Another possible reason for the apparent species difference with this drug is involved with its effect on enzyme systems. The likelihood of its potent monamine oxidase inhibitory activity has been mentioned already as has also the possibility of its block of norepinephrine synthesis through inhibition of the production of the norepinephrine precursor, dihydroxyphenylethylamine (3, 4). Since inhibition of monamine oxidase in the body results in an elevation of both norepinephrine and serotonin levels, this effect obviously cannot explain the blood pressure-lowering activity in humans. If, however, the effect to inhibit norepinephrine synthesis were relatively the more prominent in the human and relatively the less prominent in the dog, this would account for the species difference. It is possible that the decrease in dihydroxyphenylethylamine production is a result of dihydroxyphenylalanine decarboxylase inhibition. The latter mechanism would seem to be a remote possibility at best, however, inasmuch as doses needed to inhibit DOPA decarboxylase far exceed those which, employed clinically, achieve an antihypertensive effect.

SUMMARY

1-Phenyl-2-hydrazinopropane has been administered to both conscious and anesthetized dogs in an attempt to determine whether any differences in blood pressure response occur between the two. No significant difference was observed.

Also, this hydrazine derivative was given both orally and intravenously to the conscious animal in an attempt to determine whether any differences in blood pressure response accrue to differences in method of administration. For the oral work, daily doses of 1 mg. per Kg. were given over a period of fourteeen days, during which both supine and standing blood pressures were recorded at intervals. The blood pressure response to the compound administered orally was qualitatively like that observed with intravenous doses.

It is a possibility that the recorded differences in blood pressure response to this agent in the human and in the dog are explained in part by the fact that the marked depressor response of the human occurred only in the hypertensive individual while the pressor response of the dog occurred in the normotensive animal.

Another possible explanation of the apparent, species-related differences in blood pressure response to this agent is that in the human the compound's action to inhibit norepinephrine synthesis dominates its action to inhibit norepinephrine destruction, while in the dog the reverse is true.

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Isolation of a β-Sitosterol Complex from *Salvia sclarea*

By HAROLD J. NICHOLAS

***Salvia sclarea*, a member of the Labiatae family, has been reported previously to contain the diterpene sclareol and two acidic pentacyclic triterpenes. The presence of free β-sitosterol is now reported, characterized unequivocally by the preparation of acetate, benzoate, dinitrobenzoate, and hydrogenated acetate. An accompanying contaminant, probably a sterol, has not yet been identified.**

THE LABIATE *S. sclarea* contains the bicyclic diterpene sclareol (1) and at least two acidic pentacyclic triterpenes (2). We now wish to report the isolation of β-sitosterol from this interesting plant, which thus provides a unique opportunity to study the simultaneous biogenesis of diterpene, sterol, and triterpene in the same organism. The sterol occurs in combination with a difficultly-separable minor constituent, which finding is not uncommon for sitosterol. The presence of β-sitosterol in *S. sclarea* was not detected in our previous investigation (2) because the crude preparations were less firmly held on alumina than the sitosterol from the other labiates examined. Purification of the crude sterol mixture as the free compound, acetate, or benzoate did not give analytical results satisfactory for proper identification. Separation satisfactory for large-scale work was achieved through preliminary fractionation of a crude 3,5-dinitrobenzoate mixture.

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EXPERIMENTAL[1]

Materials.—Ground *Salvia sclarea* was obtained from the Wunderlich-Diez Corporation, Hasbrouck-Heights, N. J. Additional source material was obtained from the S. B. Penick Company, New York, N. Y., as Solid Extract Clary Sage (unadjusted), representing the total extractives of Clary Sage extracted with 95% ethanol. Solvents for chromatography were A. R. grade distilled before use. The alumina was Fisher grade, 80–200 mesh, for chromatography.

Separation of Sterol Mixture.—The ground plant was exhaustively extracted with hot ethanol and the extract was concentrated *in vacuo* to a thick gum.[2] This (or the Clary Sage extract freed of ethanol *in vacuo*) was extracted with hot ligroin and the latter (cooled) was extracted with 1% aqueous potassium hydroxide to remove any residual acidic triterpene. The ligroin was then washed with water and distilled to dryness *in vacuo*.

The ligroin residue (30 Gm.) was dissolved in 200 ml. warm benzene and poured onto a 300-Gm. (6 cm. diameter) alumina column previously washed with benzene. Benzene (3,000 ml.) was then poured through the column; the eluate on distillation gave

[1] Microanalyses were performed by Dr. G. Weiler and Dr. F. B. Strauss, Microanalytical Laboratory, Oxford, England. All optical rotations were determined in chloroform. Melting points were obtained on a Fisher-Johns hot stage apparatus.
[2] Subsequent steps were applicable to the nonsaponifiable fractions of alcohol extracts of the plant.

12.0 Gm. deep red oil. A crude sterol mixture could be separated from this oil by the addition of methanol or by precipitation with digitonin. Equally facile, however, was an additional chromatography (see below). Subsequent washing of the column with 2% ethanol in benzene yielded 18.0 Gm. of greenish semicrystalline oil representing largely crude sclareol (2). The column was then discarded.

Further Purification of Sterol Mixture.—The deep red oil (above, 19 Gm.) was dissolved in 200 ml. of petroleum ether (b. p. 30–60°) and poured onto a 200 Gm. petroleum ether-washed column (6 cm. diameter). Addition of petroleum ether until the eluates were nearly colorless (about 2,000 ml.) was followed by addition of benzene aliquots; yields are given in Table I.

TABLE I.—YIELD OF CRUDE STEROL MIXTURE

Fraction No.	Volume C_6H_6, ml.	Material Eluted, Gm.
1	200	2.3, Red oil
2	200	1.8, Red oil
3	200	0.5, Red oil
4	200	0.1, Red gum
5	200	0.1, Red gum
6	200	0.3, Red gum
7	200	0.1, Red gum
8	200	0.3, Red gum
9	1,000	0.5, Red gum
10	1,000	0.6, Red gum
11	1,000	0.3, Yellow, crystalline solid
12	2,000	0.2, Yellow, crystalline solid

Fractions 11 and 12 were combined and crystallized twice from acetone-methanol giving 0.4 Gm. white rods, m. p. 138–140°.[1] Additional material of lesser purity (5.0 Gm., m. p. 132–140°) was obtained from combined fractions 1 to 10 by three crystallizations from acetone-methanol. Neither of these preparations exhibited selective absorption in the region 220–300 mμ in absolute ethanol. Preparation of an acetate bromide using 3 Gm. of crude sterol gave no indication of insoluble stigmasterol acetate tetrabromide. Preparation of an acetate, benzoate, and a stanol from this crude sterol fraction indicated the probability that the principal component was β-sitosterol, but the analytical data were not satisfactory.

Purification Through the 3,5-Dinitrobenzoate.—Crude sterol (m. p. 138–140°, 7.5 Gm.) was refluxed for forty-five minutes with 15 Gm. 3,5-dinitrobenzoyl chloride and 85 ml. anhydrous pyridine. The cooled mixture was diluted with water, extracted thoroughly with ethyl ether and the latter washed successively with water, 10% hydrochloric acid, 1% sodium hydroxide, and water. Distillation of the ether gave 6.0 Gm. of reddish solid. This was dissolved in an excess of hot ethyl acetate. On cooling, 5 Gm. of irregular crystals precipitated, m. p. 205–209°. This will be referred to as the "difficultly soluble 3,5-dinitrobenzoate." No further crystallization occurred in the filtrate after several days standing. On reducing to low volume under nitrogen (steam bath) and addition of a little ethanol, 1.0 Gm. of pink needles were obtained, m. p. 197–200°. Examination of this "easily-soluble" 3,5-dinitrobenzoate is in progress.

Difficultly-Soluble 3,5-Dinitrobenzoate.—Preparation of β-Sitosterol and Derivatives.—After several crystallizations from hot ethyl acetate the difficultly soluble 3,5-dinitrobenzoate was saponified with 5% alcoholic potassium hydroxide. The free compound so obtained gave needles, m. p. 140–141° after two crystallizations from acetone-methanol. This material was used to prepare the following derivatives.

β-Sitosteryl Acetate.—The above free compound (2.49 Gm.) was acetylated in pyridine with acetic anhydride (0.5-hour reflux). The product after two crystallizations from acetone-methanol gave 1.50 Gm. of scales, m. p. 126–128°, undepressed on admixture with β-sitosteryl acetate, and unchanged after several additional crystallizations from ethyl ether-methanol; $[\alpha]_D^{25}$ −35.5°.

Anal.—Calcd. for $C_{31}H_{52}O_2$: C, 81.52; H, 11.48. Found: C, 81.56; H, 11.26. Weizmann, *et al.* (3), give m. p. 129–130°, $[\alpha]_D^{18}$ −44° for β-sitosteryl acetate.

β-Sitosteryl Benzoate.—The free compound (0.22 Gm.) was treated with benzoyl chloride in pyridine (0.5-hour reflux). The product on work-up was crystallized two times from acetone, giving 0.12 Gm. plates, m.p. 147–149°, undepressed on admixture with β-sitosteryl benzoate. Three additional crystallizations from methylene chloride-methanol gave plates, m. p. 149–150°; $[\alpha]_D^{21}$ −9.07°.

Anal.—Calcd. for $C_{36}H_{54}O_2$: C, 83.34; H, 10.49. Found: C, 83.32; H, 10.40. Weizmann, *et al.* (3), give m. p. 146–147° and $[\alpha]_D^{18}$ −12° for β-sitosteryl benzoate.

β-Sitosterol.—The acetate (m. p. 126–128°, 0.9 Gm.) was saponified with 5% alcoholic potassium hydroxide. The saponified product after three crystallizations from ether-methanol gave needles, 0.6 Gm., m. p. 140–141°; $[\alpha]_D^{25}$ −28.7°.

Stigmastanol Acetate.—The above acetate (m. p. 126–128°, 0.24 Gm.) in 50 ml. glacial acetic acid was shaken under 50 psi hydrogen pressure for three hours with 50 mg. platinum oxide. After removal of the acetic acid *in vacuo* the product was crystallized three times from ethyl ether-methanol, giving needles, m. p. 137–139°; $[\alpha]_D^{18}$ +17.7°, undepressed on admixture with an authentic sample of stigmastanol acetate prepared by hydrogenation of stigmasteryl acetate.

Anal.—Calcd. for $C_{31}H_{54}O_2$: C, 81.16; H, 11.87. Found: C, 81.25; H, 11.78. Weizmann, *et al.* (2), give m. p. 134–136°; $[\alpha]_D^{18}$ +14° for stigmastanol acetate.

Stigmastanol.—Saponification of the above stigmastanol acetate with 5% alcoholic potassium hydroxide and several crystallizations of the product so obtained from ethyl ether-methanol gave rods, m. p. 142–144°; $[\alpha]_D^{25}$ +26.0°.

β-Sitosteryl Chloride.—The above free compound (m. p. 139–140°, 0.189 Gm.) was treated with phosphorus chloride (0.60 Gm.) in 100 ml. heptane, according to Djerassi, *et al.* (4), and the resulting product was chromatographed on an acetic acid-deactivated alumina column (10 Gm.). Three 25-ml. fractions of heptane removed 0.148 Gm. of white solid which on four crystallizations from acetone-

[1] The same crude product was obtained from nonsaponifiable fractions of alcohol extracts of the plant by precipitation with digitonin and recovery of sterol from the digitonide.

methanol or acetone-ethanol gave rods, m. p. 85–86°. Shoppee (5) gives m. p. 88–89° for β-sitosteryl chloride.

Anal.—Calcd. for $C_{29}H_{49}Cl$: Cl, 8.18. Found: Cl, 8.15.

β-Sitosterol 3,5-Dinitrobenzoate.—The free compound (0.26 Gm.) was refluxed 0.5 hour with 0.60 Gm. 3,5-dinitrobenzoyl chloride in 8 ml. anhydrous pyridine. The product, worked-up as described for the crude dinitrobenzoate mixture, gave light yellow needles after four crystallizations from ethyl acetate, m. p. 208–209°; $[\alpha]_D^{25}$ −12.0°.

Anal.—Calcd. for $C_{43}H_{62}N_2O_6$: C, 71.02; H, 8.61. Found: C, 71.20; H, 8.68. Weizmann, *et al.* (3), give m. p. 200–201°, $[\alpha]_D^{15}$ −11° for β-sitosterol *m*-dinitrobenzoate.

DISCUSSION

Ruzicka has suggested on theoretical grounds (6) that the plant pentacyclic triterpenes as well as the plant sterols have a common biosynthetic origin through the selective folding of the squalene molecule. One experimental approach to this problem should be through the administration of an appropriate C^{14}-labeled precursor of squalene and subsequent comparison of the specific activities of the isolated sterols and triterpenes. An approach to this problem has already been made (7). Several members of the Labiatae family seem suitable for this type of study (2). On the basis of the present work, *S. sclarea* may be included in this group since it contains oleanolic acid, ursolic acid II,[4] and β-sitosterol. Since the bicyclic diterpene sclareol is also present in this plant, the latter provides a rather unique opportunity to study the biosynthesis of several polyisoprenoid substances within the same organism. In preliminary experiments in which 2-C^{14}-sodium mevalonate was absorbed by cut stems of flowering *S. sclarea* there was considerable incorporation of C^{14} into digitonin-precipitable material and the acidic triterpene fraction of extracts of the plant. It was of interest that the incorporation of C^{14} into sclareol under these circumstances was very low(8).

For tracer experiments the purification of sterol and triterpene and the nature of allied contaminants becomes of critical importance. Investigation is in progress to determine the identity of the sterol (?) which accompanies β-sitosterol in *S. sclarea*.

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[4] The constitution of this triterpene has not yet been determined.

Analysis of Acetylsalicylic Acid and Acetylsalicylic Acid Tablets

By JOSEPH LEVINE

The U. S. P. assays for acetylsalicylic acid and its tablets are not universally applicable to these dosage forms containing buffers, flavoring agents, coloring materials, and enteric coatings. The U. S. P. test for free salicylic acid is not quantitative and may be subject to error unless additional controls are provided. A method of assay is presented which is applicable to tablets containing the usual excipients used in the preparation of plain, buffered, enteric coated, and children's tablets. A quantitative procedure for the determination of free salicylic acid is also recommended.

THE U. S. P. assay of acetylsalicylic acid tablets (1*a*), which is based upon the determination of the ester value of the neutralized sample, is not applicable to many formulations containing buffers, flavoring agents, and coloring substances. The buffering components and aluminum salts preclude the neutralization of the buffered tablets and aluminum aspirin, and the ester components of the flavored tablets may affect the results.

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A procedure has been described (2) for the determination of acetylsalicylic acid in mixtures with acetophenetedin and caffeine, in which the acetylsalicylic acid is isolated by modified partition chromatography and measured spectrophotometrically. By appropriate simplification, this procedure has been adapted to the assay of acetylsalicylic acid tablets, including commercial buffered tablets, the colored and flavored children's tablets, tablets of aluminum aspirin, and enteric-coated tablets. An aliquot of the sample of acetylsalicylic acid or the ground tablets dissolved in chloroform is passed over a

chromatographic column consisting of sodium bicarbonate adsorbed on Celite. The acetylsalicylic acid is trapped on the column while the other chloroform-soluble materials pass through into the eluate. The acetylsalicylic acid is eluted with a chloroform-acetic acid solution and determined spectrophotometrically. Free salicylic acid will accompany the acetylsalicylic and, in concentrations of 5 per cent or more, can be determined simultaneously (2, 3).

A small quantity of hydrochloric acid, dissolved in methanol to achieve miscibility with chloroform, is added to the sample solution to stabilize the acetylsalicylic acid. The instability of acetylsalicylic acid in chloroform has been noted (2). It has been found that two reactions occur: the hydrolysis of the acetyl group, producing salicylic acid; and the formation of acetylsalicylic anhydride (4). Anhydride formation is greatly accelerated in the presence of weakly alkaline reagents, such as magnesium carbonate, a component of some buffered aspirin tablets. Small concentrations of acetic acid are effective in preventing anhydride formation but, because of the limited ionization of acetic acid in chloroform, do not protect adequately against the hydrolytic effect of the buffering agents. Hydrochloric acid effectively prevents both reactions, and the quantity needed for this protection is sufficiently small that it does not adversely affect the operation of the chromatographic column.

The U. S. P. test for free salicylic acid in acetylsalicylic acid (1*b*) and its tablets (1*a*) is not quantitative. It is simply a limit test in which the colors developed with ferric ion by the sample and by a salicylic acid standard are compared visually. Strode, *et al.* (5), commented critically on this procedure and outlined conditions for obtaining precise spectrophotometric and visual measurement, the latter being limited to colorless samples. Their procedure requires close regulation of temperature and time, and provides for a correction based upon the rate of hydrolysis of acetylsalicylic acid. They correct for the effect of acetylsalicylic acid upon the color of the ferric ion-salicylic acid complex by adding pure acetylsalicylic acid to the salicylic acid standard. The U. S. P. test does not provide for this correction.

Concentrations of free salicylic acid in the order of 5 per cent or higher can be determined directly and simultaneously with the determination of acetylsalicylic acid by measurement of absorbance at 310 mμ. This procedure is not sensitive enough for concentrations of free salicylic acid in the range of the U. S. P. or N. F. requirements.

The isolation of salicylic acid would permit its direct spectrophotometric determination, uncomplicated by any of the factors which affect the ferric ion-salicylic acid color or the hydrolytic effect of the reagent on the sample. The separation of small amounts of approximately equal concentrations of salicylic and acetylsalicylic acids by paper electrophoresis and chromatography has been reported (6), but this procedure cannot be applied to the isolation of sufficient salicylic acid for spectrophotometric measurement from a mixture in which it occurs in a concentration in the order of 1:1000.

Salicylic acid can be removed from chloroform solution by dilute aqueous solutions of ferric chloride, while acetylsalicylic acid, lacking the phenolic hydroxyl group, remains in the chloroform. In passing a chloroform solution of the sample over a partition chromatographic column on which ferric chloride is incorporated as stationary phase, salicylic acid is trapped, leaving a purple zone on the column, while acetylsalicylic acid passes through. Contact of the acetylsalicylic acid with the aqueous phase is transitory, allowing minimal time for hydrolysis to occur. Acetic acid in chloroform solution dissociates the ferric ion-salicylic acid complex. The liberated salicylic acid is eluted and determined spectrophotometrically.

The concentration of ferric chloride greatly affects the formation of the ferric ion-salicylic acid complex. Very dilute solutions of ferric chloride remove salicylic acid from chloroform readily. As the concentration of ferric chloride increases, the completeness of the removal decreases. A ferric chloride solution of the strength of the U. S. P. test solution (1*c*) is wholly ineffective. Since a chloroform solution of acetylsalicylic acid removes a large quantity of iron, presumably as ferric acetylsalicylate, from the column a sufficient quantity of ferric chloride must be present to avoid depletion. Thus, a balance must be maintained between a sufficiently low concentration of ferric chloride to trap the salicylic acid and a sufficiently large quantity of ferric chloride to provide an excess over the amount removed by the acetylsalicylic acid. For a sample of 200 mg. acetylsalicylic acid, an effective balance is achieved by the use of 5 Gm. of Celite with 7 ml. of 2 per cent ferric chloride solution. This ratio of aqueous phase to Celite is greater than is customarily used, but it introduces no complications. Under these conditions, using the volumes of solvent de-

scribed in the experimental section, the salicylic acid migrates to approximately 1 cm. from the bottom of the column by the time the acetylsalicylic acid has been completely removed.

The procedure was applied to pink acetylsalicylic acid tablets, enteric-coated aspirin tablets, orange-colored and flavored children's aspirin tablets, aluminum aspirin tablets, and buffered aspirin tablets. The coloring matter remains at the top of the column throughout the assay in all cases, and offers no difficulties. It the case of the buffered aspirin and the aluminum aspirin, the acetylsalicylic acid present as the chloroform-insoluble salt must be transformed to the free acid in order that it can be eluted in the proper fraction. Acetic acid cannot be used for this purpose since it dissociates the ferric ion-salicylic acid complex. Boric acid, however, affects the transformation without affecting the functioning of the column.

A slight modification of the procedure was required for enteric-coated tablets. The surface-active agents present in these tablets affects the performance of the chromatographic column by stripping the aqueous phase off with both the prewash and the salicylic acid eluate. By passing the sample solution over a separate column containing dry Celite directly prior to its passage over the ferric chloride column, the interfering components are removed. This preliminary column is advantageous in the case of children's tablets also, serving as a filter for the great bulk of excipient material.

EXPERIMENTAL

Preparation of Standards.—*Acetylsalicylic Acid.*—Transfer about 50 mg., accurately weighed, of pure material to a 50-ml. volumetric flask, add 30 ml. of chloroform and 0.5 ml. of glacial acetic acid, and adjust to volume with chloroform. Transfer 5.0 ml. of this solution to a 100-ml. volumetric flask, add 1 ml. of acetic acid, and adjust to volume with chloroform. Use within eight hours. The absorbance of this solution at 280 mμ is approximately 0.405. The absorbance at 310 mμ should be approximately 0.003. If it is appreciably greater than this, purify the acetylsalicylic acid by recrystallization from isopropanol.

Salicylic Acid.—Dissolve about 25 mg., accurately weighed, of pure material in 100 ml. of chloroform. Transfer 5.0 ml. to a 100-ml. volumetric flask, add 1 ml. of glacial acetic acid, and adjust to volume with chloroform. The absorbances of this solution at 310 and 280 mμ are approximately 0.374 and 0.074, respectively.

Chromatographic Column.—Use a 25 × 200 or 250 mm. test tube to which is attached an approximately 50 mm. length of 6–8 mm. tubing. The tamping rod consists of a disk of stainless steel, aluminum, or glass, of a diameter 1 mm. less than that of the column, attached to a rod 12 to 18 inches long. Pack fine glass wool[1] in the column as support.

Procedure.—*Acetylsalicylic Acid.*—To 3 Gm. Celite 545[2] add 2 ml. of 1 *N* sodium bicarbonate solution and incorporate thoroughly by kneading with a flexible spatula blade. Transfer to column and tamp, using gentle pressure, to a uniform mass.

Weigh a sufficient number of tablets to provide a representative sample and grind to a fine powder. To a 50-ml. volumetric flask containing 1 ml. of a solution of hydrochloric acid in methanol (1 + 49), transfer a weighed sample containing approximately 50 mg. of acetylsalicylic acid with the aid of 40 ml. of chloroform, shake thoroughly, and adjust to volume with chloroform.

(Use water-washed chloroform in the following steps.) Pass 5.0 ml. of the sample solution onto the column, and wash successively with 5-ml. and 25-ml. portions of chloroform. Place a 100-ml. volumetric flask under column as receiver and elute with a solution of 0.5 ml. of acetic acid in 5 ml. of chloroform, followed by 94 ml. of 1% acetic acid in chloroform. Adjust to volume, and determine the absorbance at 280 and 310 mμ. Compare these values with those determined simultaneously for standard solutions of acetylsalicylic acid and of salicylic acid.

When the absorbance of the sample solution at 310 mμ is 0.075 or higher, it represents a concentration of 5% or more of free salicylic acid. Under these conditions, the amount of free salicylic acid, in mg., in the sample taken can be calculated from the formula: $0.5 \times w_2 \times A_{u(310)}/A_{s(310)}$, where w_2 is the weight of salicylic acid used in preparing the standard solution and $A_{u(310)}$ and $A_{s(310)}$ are the absorbances of the sample and salicylic acid standard solutions, respectively, at 310 mμ.

To calculate the amount of unhydrolyzed acetylsalicylic acid, the observed absorbance of the sample at 280 mμ must be corrected by the formula: $A_{u(280)\ \text{corrected}} = A_{u(280)} - A_{u(310)} \times (A_{s(280)}/A_{s(310)})$, where A_u and A_s are the absorbances of the sample and salicylic acid standard solutions, respectively, at the designated wavelengths. The amount of intact acetylsalicylic acid, in mg., in the sample taken equals $w_1 \times A_{u(280)\ \text{corrected}}/A_{s(280)}$, where w_1 is the weight of acetylsalicylic acid used in preparing the standard solution and $A_{s(280)}$ is the absorbance of that solution at 280 mμ.

Salicylic Acid (For Samples Containing Less than 5%).—To 5 Gm. Celite 545 add 7 ml. of ferric chloride solution (1 in 50)[3] and incorporate thoroughly by kneading with a flexible spatula blade. Transfer to column and tamp to a uniform mass, using gentle pressure. Weigh a quantity of sample containing 200 mg. of acetylsalicylic acid into a beaker and add 10 ml. of chloroform. (For samples of buffered aspirin or aluminum aspirin, first add 0.25 ml. of 10% solution of boric acid in methanol.) Swirl one minute to dissolve the acetylsalicylic acid, and transfer to the column, using 5 ml. of chloroform, in several small amounts, to rinse container. Wash the column successively with 10 ml. and 25 ml. of chloroform to remove acetylsalicylic acid. The initial effluent will be so concentrated that frequently

[1] Pyrex Filtering Fiber, Corning Glass Catalogue No. 3950.
[2] Johns-Manville Corp.
[3] Ferric chloride solutions hydrolyze on standing, becoming cloudy; use only a clear solution.

TABLE I.—ANALYSIS OF COMMERCIAL ACETYLSALICYLIC ACID TABLETS

Sample	Acetylsalicylic Acid, Grains per Tablet Labeled	Found	Salicylic Acid Found, %[d]
Acetylsalicylic acid tablets	5	4.89, 4.89[a]	0.06
		4.89, 4.86	0.07
Children's aspirin tablets (colored and flavored)	1.25	1.28, 1.28	0.09
		1.27, 1.27	0.10
Buffered aspirin tablets	5	4.87, 4.87	0.41
		4.86, 4.86	0.39
Pink acetylsalicylic acid tablets	5	5.01, 5.02	0.14
		5.00, 4.98	0.13
Aluminum aspirin tablets, children's (colored and flavored	1.25	1.08, 1.08[b]	10.1[e]
		1.08, 1.08	10.3[e]
		1.24, 1.24[c]	10.0, 10.1[f]
			10.1, 10.1[f]
Enteric-coated acetylsalicylic acid tablets	5	5.09, 5.09	0.24
		5.06, 5.03	0.24

[a] Consecutive figures denote analyses of replicate aliquots of a sample solution. [b] Value for intact acetylsalicylic acid. [c] Total of free salicylic acid, calculated as acetylsalicylic acid, plus intact acetylsalicylic acid. [d] Salicylic acid is expressed in per cent of the declared amount of acetylsalicylic acid. [e] Determined by procedure for salicylic acid. [f] Determined by procedure for acetylsalicylic acid.

acetylsalicylic acid will crystallize on the tip of the outlet tube and should be removed with a jet of chloroform or alcohol. Note the position of the purple ferric ion-salicylic acid zone during the washing. With high concentrations of salicylic acid this may reach the bottom of the column with resultant loss of salicylic acid. If this occurs, repeat the assay with a smaller sample.

For enteric coated tablets or children's aspirin tablets, transfer to a separate column 3 Gm. of dry Celite, and mount in such a manner that effluent from this column passes directly onto the ferric chloride column. Place a loose plug of glass wool above the ferric chloride-Celite in the latter column to prevent channeling. Transfer the sample and wash as above. Discard the upper column and wash the ferric chloride column with an additional 10 ml. of chloroform.

Place a 50-ml. volumetric flask under column as receiver. Pass 5 ml. of chloroform containing 0.5 ml. of glacial acetic acid, followed by 44 ml. of 1% acetic acid in chloroform. Adjust to volume and determine the absorbance at 310 mμ. The quantity of salicylic acid, in mg., in the sample taken is equal to $w(A_u/A_s)$, where w is the weight, in mg., of salicylic acid in 50 ml. of the standard solution and A_u and A_s are the absorbances of the sample and standard solutions, respectively, at 310 mμ.

DISCUSSION

Pure acetylsalicylic acid was prepared by twice recrystallizing the U. S. P. product from isopropanol. The salicylic acid isolated from 200 mg. of this material by the proposed procedure yielded an absorbance of 0.003 at 310 mμ, equivalent to less than 0.003%. The salicylic acid isolated from a 200-mg. sample of the pure acetylsalicylic acid to which had been added 280 mcg. of salicylic acid had an absorbance of 0.170 at 310 mμ as compared with a value of 0.169 given by the same quantity of salicylic acid measured directly.

A variety of commercial acetylsalicylic acid tablets were analyzed by the above procedures. The results are presented in Table I.

SUMMARY

Procedures are presented for the determination of acetylsalicylic acid and of small concentrations of free salicylic acid in acetylsalicylic acid tablets, buffered tablets, enteric coated tablets, aluminum aspirin tablets, and colored and flavored tablets. Acetylsalicylic acid is isolated by passage of a chloroform solution of the sample over a chromatographic column in which sodium bicarbonate on Celite is the stationary phase, followed by neutralization of the stationary phase *in situ*, and elution with acetic acid in chloroform. Salicylic acid is separated from acetylsalicylic acid by trapping it as the ferric ion-salicylic acid complex in a chromatographic column. The separated salicylic acid is isolated by dissociation of the complex and elution with acetic acid in chloroform. The isolated acetylsalicylic and salicylic acids in the eluates are measured spectrophotometrically.

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Physicochemical and Biological Potency Relations for Isomerized Vitamin A Palmitate

By ELMER DE RITTER

Vitamin A losses by the U. S. P. spectrophotometric assay due to isomerization of all-*trans* vitamin A during storage of aqueous multivitamin drops average 7 per cent. These losses are over and above the actual degradation losses measured by the Carr-Price method which is not influenced by isomerization. This 7 per cent loss can be avoided by use of preisomerized vitamin A but, in either case, precise rat liver storage assays yield lower potencies after isomerization than U. S. P. assays. The data in this report confirm formation of the less active 9-*cis* or 9,13-di-*cis* isomers as reported by Lehman, *et al.* (1), and Ames, *et al.* (2), with their sources of vitamin A. With other sources of vitamin A, the extent of isomerization at the 9-position is in many cases less than would be indicated by the cubic regression equation proposed by these workers. Hence, the present data do not support the general applicability of Ames' equation for all commercial sources of vitamin A. A need remains for a reliable method or routine determination of each of the vitamin A isomers. In the meantime, biopotency can be estimated by an empirical correction of the U. S. P. assay based on the maleic value.

ALTHOUGH EXTENSIVE isomerization of vitamin A during storage of aqueous multivitamin preparations had been recognized for some years via the maleic anhydride reaction, only recently have the physicochemical and biological investigations of Lehman, *et al.* (1), and Ames, *et al.* (2), revealed that isovitamin A_a (9-mono-*cis*) and isovitamin A_b (9,13-di-*cis*) are formed also in appreciable quantities in addition to neovitamin A_a (13-mono-*cis*). A cubic regression equation was proposed by Ames, *et al.* (2), to calculate biopotency from the Carr-Price assay and maleic value. The present communication summarizes extensive stability tests of various sources of commercial, all-*trans* and preisomerized vitamin A palmitate in aqueous multivitamin drops by U. S. P. spectrophotometric, Carr-Price, and rat liver storage assays. The data define the average loss of U. S. P. spectrophotometric potency due to isomerization. They confirm fairly well the applicability of the regression equation of Ames, *et al.* (2), to aged multivitamin preparations made with preisomerized vitamin A, but do not confirm its applicability to initial assays of other sources of preisomerized vitamin A or to aged multivitamin preparations made with another source of all-*trans* vitamin A palmitate. In the latter instances, the biopotencies indicate a much lower content of 9-mono-*cis* or 9,13-di-*cis* isomers.

EXPERIMENTAL

An extensive series of stability tests has been carried out with samples of synthetic vitamin A palmitate, including essentially all-*trans* products from four manufacturers and preisomerized material from three of these sources, to determine the performance of these products in typical aqueous multivitamin drop formulations. The pH levels of the experimental drops[1] were adjusted to 3.5 or 5.0 to cover the pH range of most commercial preparations. Since the differences in vitamin A stability to be measured were relatively small, samples of the multivitamin drops were stored in sealed, amber ampuls to avoid possible differences in stability due to variations in tightness of closure of screw-cap bottles. To minimize analytical variation, assays were run at least in triplicate with only one assay on a given sample per day. Carr-Price (antimony trichloride or blue-color) assays were run against the U. S. P. Vitamin A Reference Standard to determine total vitamin A, since all 4 isomers yield equal values by this method (3). The maleic anhydride reaction was carried out as described by Robeson and Baxter (4) to measure amounts of unreactive 13-mono-*cis*- and 9,13-di-*cis* isomers. Maleic values were calculated as described by Ames, *et al.* (2).

Rat liver storage bioassays were run with two levels of U. S. P. Reference Standard solution and of test samples plus an undosed control in each assay. From 30 to 60 rats were used at each level of standard and sample to minimize the limits of error of the bioassays. Vitamin A concentrates and the U. S. P. Standard were diluted in oil to the dosage levels and 0.1 ml. administered orally by syringe. Multivitamin drops were administered directly by the same method after it was ascertained that dosage of vitamin A in oil or in the drops yielded equivalent liver storage of vitamin A. Levels of about 500 and 1,000 units were dosed on two successive days, and on the fifth day the livers were removed for assay by the Carr-Price method. Potencies were calculated by the slope-ratio procedure described by Ames and Harris (5).

RESULTS AND DISCUSSION

Vitamin A palmitate concentrates with maleic values ranging from the 3 to 6% of commercial,

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[1] Made with the composition of Vi-Penta Drops No. 3, Hoffmann-LaRoche.

TABLE I.—STABILITY OF VITAMIN A PALMITATE IN AQUEOUS MULTIVITAMIN DROPS

Initial Maleic Value,	Loss of Vitamin A, %			
	At 45°		At Room Temperature	
	6 wk.	9 wk.	6 mo.	12 mo.
	Carr-Price Assay			
3-6	7	9	4	8
18-25	6	7	4	6
28-35	7	9	5	8
	U. S. P. Spectrophotometric Assay			
3-6	16	20	10	15
18-25	14	15	8	11
28-35	10	13	6	7

all-*trans* products to the 28-35% of preisomerized "equilibrium mixtures" have been incorporated into aqueous multivitamin drops and stored for six and nine weeks at 45°, and for six and twelve months at room temperature. A summary of vitamin A losses by both Carr-Price and U. S. P. spectrophotometric assays is given in Table I. These data represent averages for 27 lots of vitamin A in 54 lots of multivitamin drops. Losses of vitamin A by Carr-Price assay are not influenced by the isomer composition of the vitamin A source. By the U. S. P. spectrophotometric assay, however, isomerization of the all-*trans* vitamin A at both 45° and room temperature leads to potency losses about 7% greater than those found when preisomerized equilibrium mixtures are used in manufacturing the multivitamin drops. This isomerization loss is due to the fact that the U. S. P. potencies of the 13-mono-*cis*, 9-mono-*cis*, and 9,13-di-*cis* isomers are only 72, 69, and 73%, respectively, of that of all-*trans* vitamin A (4, 6, 7). The maximum maleic value of about 33% was found in all cases after six weeks at 45° and twelve months at room temperature when the multivitamin drops were made with all-*trans* vitamin A. In three weeks at 45° or six months at room temperature, a maleic value of about 25% was found.

The effect of isomerization on the biological potency of the vitamin A was determined by rat liver storage bioassays. Although the biopotencies of the 13-mono-*cis*, 9-mono-*cis*, and 9,13-di-*cis* isomers have been reported as 105, 33, and 31%, respectively, of the corresponding U. S. P. spectrophotometric values (4, 6, 7), there is no convenient physicochemical method for determining the levels of each of the individual isomers in an isomerized product which would permit a direct calculation of biopotency. For this reason biopotencies have been evaluated directly by rat bioassays. Table II shows the results of a typical bioassay and the corresponding physicochemical assays of two samples of aqueous multivitamin drops made with all-*trans* vitamin A palmitate after storage for twenty-seven and twenty-two months at room temperature, which is about the maximum shelf life of such products. In both cases the biopotency is significantly less than the U. S. P. assay, indicating appreciable isomerization to the 9-mono-*cis* and/or 9,13-di-*cis* isomers.

The overall influence of this isomerization on the various bioassays is summarized in Fig. 1 in which a comparison is made also with the line defined by

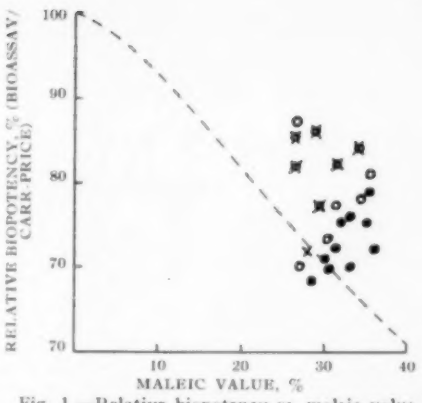

Fig. 1.—Relative biopotency *vs.* maleic value of isomerized vitamin A palmitate. Initial assays of preisomerized concentrates: (⊠) AQUAPALM Hoffmann-La Roche, and VAPAD Pfizer; ×, TC MYVAX, Distillation Products Industries. Assays of aged multivitamin preparations made with: ○, all-*trans* vitamin A palmitate (*P*1.8, Hoffmann-La Roche); ●, AQUAPALM and VAPAD. - - - All-*trans* vitamin A palmitate [data of Ames, *et al.*, THIS JOURNAL, **49**, 366(1960)].

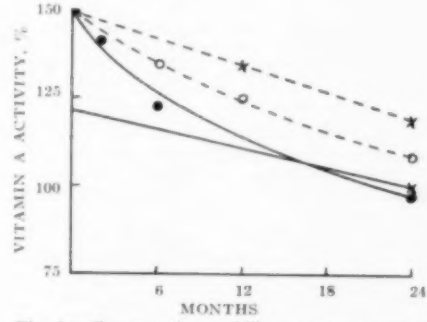

Fig. 2.—Comparative stability at room temperature of all-*trans* (○ and ●) *vs.* preisomerized (×) vitamin A palmitate in aqueous multivitamin drops made with equal potency by U. S. P., spectrophotometric assay. - - -, U. S. P. spectrophotometric assays; ——, rat liver storage bioassays.

the regression equation of Ames, *et al.* (2), for calculating biopotency from the Carr-Price assay and the maleic value. It is evident that for the present series of bioassays most of the points fall above this line. This is particularly true of the all-*trans* vitamin A[2] isomerized by storage in multivitamin drops and most of the preisomerized concentrates. In view of the fact that the limits of error of the individual bioassays at $P = 0.05$ were only ±3 to 6%, the differences from the line are in many cases highly significant. The only preisomerized vitamin A which yielded a point on the line was TC Myvax.[3]

After storage of multivitamin drops made with other preisomerized concentrates, the maleic values (Fig. 1) tend to increase slightly and the relative

[2] Hoffmann-La Roche Inc.
[3] Distillation Products Industries.

TABLE II.—ASSAYS OF AGED MULTIVITAMIN DROPS

Sample	Carr-Price, u./ml. (% Retention)	Maleic Value, %	U. S. P. Spectrophotometric Assay, u./ml. (Range at $P = 0.05$)	Rat Liver Storage, u./ml. (Range at $P = 0.05$)	Bioassay/U. S. P. Spectrophotometric Assay, %
All-*trans*[a]	8,270	35.2	7,590	6,700	88
pH 5.0	(79)	..	(7,400–7,780)	(6,500–6,900)	..
27 months/75°F.					
All-*trans*	9,530	34.6	8,230	7,480	91
pH 3.4	(85)	..	(8,110–8,340)	(7,270–7,680)	..
22 months/75°F.					

[a] Vitamin A palmitate (*P* 1.8, Hoffmann-La Roche Inc.).

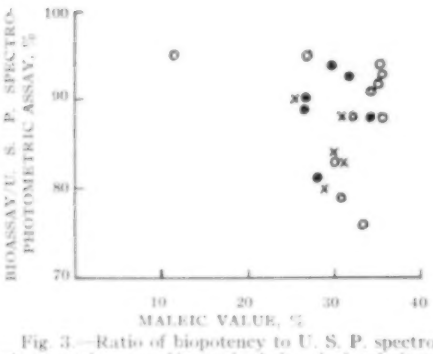

Fig. 3.—Ratio of biopotency to U. S. P. spectrophotometric assay of isomerized vitamin A palmitate. ●, Preisomerized concentrates; ○, aged aqueous multivitamin drops; ×, aged multivitamin capsules.

biopotencies fall closer to the line. It is apparent from these data that the all-*trans* vitamin A palmitate[3] does not isomerize as extensively to the lower potency 9-mono-*cis* and/or 9,13-di-*cis* isomers as the concentrates reported by Ames, *et al.* (2). Also, other preisomerized vitamin A concentrates with similar maleic values have higher relative biopotencies than those found by Ames, *et al.* (2), indicating a higher proportion of the more active 13-mono-*cis* isomer in the other preisomerized vitamin A products plotted in Fig. 1.

Although preisomerized vitamin A palmitate yields better stability in aqueous dispersions than all-*trans* vitamin A palmitate when assayed by the U. S. P. spectrophotometric method, the all-*trans* product has the advantage of a higher initial biological potency. Figure 2 shows the relative stability of both types of vitamin A by the two assay procedures in multivitamin drops stored at room temperature for two years. Although the advantage in biopotency of the all-*trans* product is reduced rapidly as isomerization progresses, it is noteworthy that even after two years, there is no significant difference in the bioassays of the two products.

The ratios of biopotency to U. S. P. spectrophotometric assay of isomerized vitamin A are summarized in Fig. 3, which includes preisomerized concentrates and all-*trans* vitamin A isomerized in multivitamin drops and soft gelatin capsules. In all cases the biopotency falls below the U. S. P. assay due to the isomerization. When the maleic value is close to the equilibrium level, the ratios fall between 76 and 95%, with an average of 88%. This range is wider than the limits of error of the assays involved and appears to indicate a significant difference in the isomer distribution in different products. Since the equation proposed by Ames, *et al.* (2), for calculating biopotency from Carr-Price assay and maleic value has been found to be inapplicable in many cases (Fig. 1), a more universally applicable method of calculation is desired. On the basis of the data in Fig. 3 and those of Lehman, *et al.* (1), which yield a slightly lower average ratio of biopotency to U. S. P. assay, an empirical equation can be set up for calculating biopotency from the U. S. P. assay and maleic value as follows:

$$\text{biopotency} = \text{U. S. P. assay} \left(1 - 0.5 \times \frac{\text{maleic value}}{100}\right)$$

Until such time as a physicochemical method suitable for routine control purposes is devised for determining amounts of all vitamin A isomers in a mixture, the above equation can serve to provide reasonable estimates of biological potency.

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Physical Chemical Approach to the Formulation of Pharmaceutical Suspensions

By ALFRED N. MARTIN

The general physicochemical principles applicable to caking and flocculation in pharmaceutical systems are reviewed and are related to practical formulations problems.

SUSPENSIONS CONSTITUTE an important class of pharmaceutical preparations. The investigation of their physical and chemical properties stands as a challenge to the industrial pharmacist and research worker because many difficult problems arise in the design and manufacture of pharmaceutical dispersions.

The kind of suspension we are concerned with here is a coarse dispersion of solid particles distributed in a liquid medium, and we must consider the properties of the suspended particles and the dispersion medium, and the interactions of the particles and the medium. In a short presentation such as this, attention can perhaps best be focused on some of the many principles and properties of suspensions by consideration of one serious problem of current interest to the research and development worker—that of caking or cementation. The principles of sedimentation, electrokinetic phenomena, and suspending techniques can, in this way, be introduced at appropriate points in the discussion. Rheology, or the flow of the suspension, and micromeritics, or small particle technology, are also considered as they pertain to the preparation and physical stabilization of a suspension.

DISCUSSION

Particle Interactions in Suspension.—By reference to Fig. 1, we see some of the factors which are concerned in the settling of particles in a suspension. The influence of gravity is constant and we can do little about it. We can, however, reduce the size of the particles and increase the consistency or rheological properties of the suspending medium and thus improve the stability by reducing the rate of settling.

But if we are to minimize caking, which I think can justifiably be called the scourge of the suspension specialist, then we must look to more subtle factors than these. Caking cannot be eliminated by reducing particle size or by increasing the consistency (yield value, viscosity, and thixotropy) of the suspending medium; indeed these measures frequently aggravate rather than prevent caking. We can approach this puzzling problem by first considering the forces of attraction and repulsion between the particles in suspension, illustrated in Fig. 1 by the diver who is forcing two particles apart.

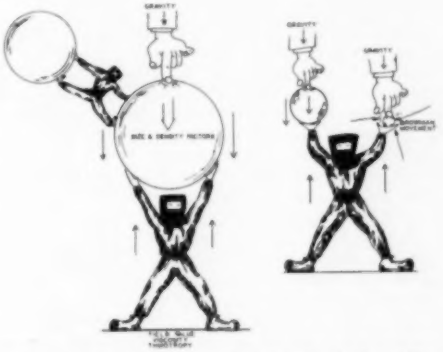

Fig. 1.—Factors influencing the physical stability of suspensions. Modified from Meyer and Cohen, *J. Soc. Cosmetic Chemists* **10**, 143(1959).

At this point in the discussion we must define several terms which are often confused or used loosely. When particles are held together strongly, we refer to these clusters as agglomerates or aggregates, and it is the aggregation of particles into a solid mass at the bottom of the container that we call caking. When particles are held together in a loose open structure in suspension, we designate these clusters as floccules or flocs, and the system is said to be in a certain state of flocculation (or deflocculation).

In Fig. 2 we see these two types of particle masses. The light fluffy flocs settle rapidly in a suspension to form a loosely arranged sediment with a large volume. Flocculated particles of sulfamerazine are shown at the right of the figure. Conversely, the individual particles in a well dispersed or deflocculated

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Some of the material for this report has been taken from the thesis of Bernard Haines, "Interfacial Properties of Powdered Materials; A Study of Caking in Liquid Dispersions," Ph.D. thesis, Purdue University, 1960. The details of experimentation and data supporting the results will be published elsewhere.

Various aspects of the general scheme proposed in this paper have been checked experimentally by Dr. Thomas Gerding, to whom the writer is grateful. Thanks are due also to Mrs. Beverly Gerding who prepared the figures included in the report.

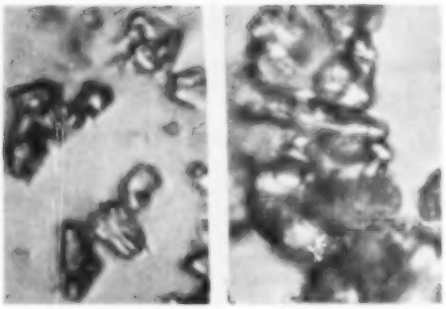

Fig. 2.—Photomicrograph showing aggregates from the caked region of a deflocculated sulfamerazine suspension (left), and the floccule structure of a sulfamerazine dispersion treated with aluminum ions (right). From Bernard Haines, Ph.D. thesis, Purdue University, 1960.

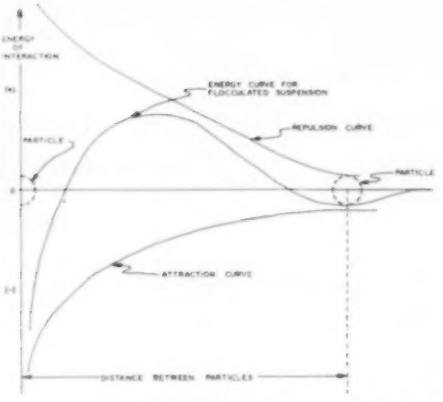

Fig. 3.—Potential energy curves for particle interactions in suspension.

lated suspension settle more slowly, but after settling they have a tendency to form a difficultly redispersible sediment or cake. Aggregated particles, removed from a sulfamerazine cake, are shown in the photomicrograph at the left of the figure. Dispersing agents, which bring about the deflocculation of a suspension, accordingly may increase the caking tendency of a dispersion, whereas flocculating agents tend to prevent it.

Flocculation and agglomeration are brought about by forces which reside at the surfaces of the particles in suspension. The particles may have ionizable groups on their surfaces or they may adsorb ions from solution to give them a negative or a positive electrical charge. Solvent molecules may also be held strongly at the particle surfaces. The charged particles are then surrounded by a cloud or ionic atmosphere of oppositely charged ions. These ions form an electrical double layer consisting of a fixed layer at the surface of the particles and a mobile layer adjacent to this stationary one and extending out into the medium. The suspended particle, together with its fixed layer, moves in an electric field, and the difference in electrical potential between the moving particle and the surrounding medium or diffuse layer is known as the zeta potential (ζ), a term we will meet again.

According to the present theory, particles in suspension are subject to two types of forces: attractive forces of the London-van der Waals type, and forces of repulsion due to interaction of the electrical double layers surrounding each particle. In Fig. 3 we find plotted the potential energy of two particles situated a definite distance apart. Here we see curves depicting the van der Waals attraction energy and the charge repulsion energy which exist when the particles are separated by various distances. The sum of the attraction and repulsion results in a third curve showing a peak and two minima or "wells."

When the repulsion energy is great the potential barrier is high and opposes collision of the particles. The system thus remains deflocculated; the particles slip past one another as they fall in the suspension, and they finally settle into a close-packed arrangement with the small particles filling the pores between the larger ones at the bottom of the container. The lowermost particles of the sediment are gradually pressed together by the weight of the particles above them. The approaching particle thus surmounts the energy barrier and rests in the energy well in close contact with its nearest neighbor. By this mechanism a deflocculated suspension frequently leads to a deposit of strongly adhering particles which are not easily resuspended by agitation.

Let us now consider flocculation. One wonders how two particles could come together to form floccules in suspension if a large potential barrier exists between them. In the case of colloidal solutions, the flocculating agent is concentrated in the double layer and reduces the repulsion of the particles. The potential barrier is lowered and the particles come together in the primary potential well. In the case of coarse suspensions, the energy barrier is too large to be surmounted during flocculation; however, a secondary minimum exists at a distance of perhaps 1,000 to 2,000 Å. separation. The particles can approach each other to this distance to yield a loosely arranged structure in suspension, as depicted in Fig. 3.

In summarizing the first portion of our discussion, we can say that flocculated particles are weakly bonded, they settle rapidly in suspension, but they are easily resuspended and do not produce a hard sediment or cake. Deflocculated particles, on the other hand, settle slowly but eventually will descend even in the presence of the most effective suspending agents, and when they settle they tend to form caked products.

Evaluating the Properties of Suspensions.—Assuming that it is desirable to produce a partially flocculated dispersion in order to prevent caking and permit easy redispersion, let us consider several techniques for studying such systems. Two methods for evaluating the degree of flocculation may be employed.

The first is known as the method of sedimentation height. It depends on measuring the final height of the sediment in graduated tubes after equilibrium has been reached. The ratio of the ultimate height H_u of sediment to the initial height H_0 of the

suspension, used as a measure of sedimentation, is shown in Fig. 4. One finds that the larger the H_u/H_0 ratio the greater is the degree of flocculation of the particles, and the ratio becomes a maximum when a sufficient concentration of flocculating agent is attained. Excess flocculant, however, may bring about a reversal in the charge on the particles and cause the system to deflocculate into individual particles. Therefore, it does not follow that if a little flocculating agent is good, a lot is better.

The second technique that we will consider briefly is that of electrophoresis. Owing to the charge on suspended particles, already referred to earlier, the particles move in an electrical field. We can observe this movement in a microelectrophoresis cell, shown schematically in Fig. 5. The particles migrate to the pole opposite in charge to that of the particle and at a velocity depending on the surface electrical charge or zeta potential. The velocity of migration of the particles is defined as the mobility in cm./sec. in a cell across which a potential of 1 v./cm. is operating. The velocity is measured by observing a particle in the microcell as it moves under a potential gradient and timing its travel with a stopwatch and a micrometer in the eyepiece of the microscope. The zeta potential is then calculated by use of the equation

$$\zeta = \frac{4\pi\eta v}{D}\,(9 \times 10^9) \cong 128 \times v$$

where ζ is the zeta potential in volts, η is the viscosity of the dispersion medium in poises, D is the dielectric constant of the dispersion medium, and v is the electrophoretic mobility in cm./sec. per volt/cm. The factor (9×10^9) converts electrostatic units into practical volts.

The Caking Diagram.—The application of sedimentation height and electrophoretic techniques are summarized in Fig. 6, where the effect of adding a negatively charged flocculating agent to bismuth subnitrate (positively charged) in suspension is shown. A similar diagram results when a negatively charged particle, such as a sulfonamide, is flocculated with a positively charged flocculating agent.

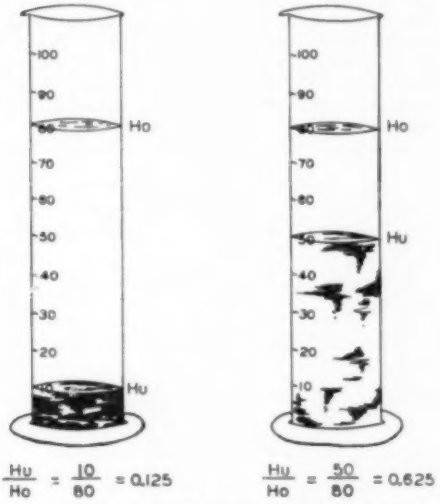

Fig. 4.—Sedimentation height as measured by the H_u/H_u ratio.

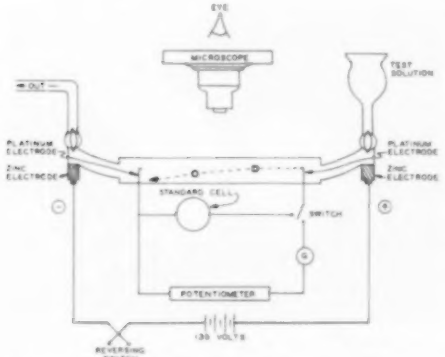

Fig. 5.—Schematic drawing of the microelectrophoresis cell.

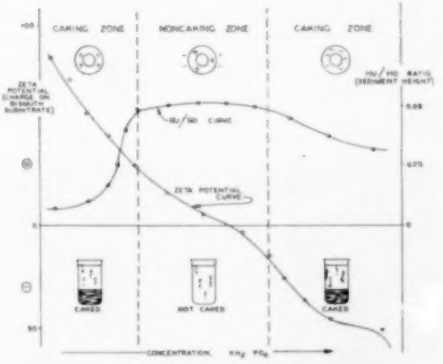

Fig. 6.—Caking diagram showing controlled flocculation of a bismuth subnitrate suspension employing dibasic potassium phosphate as the flocculating agent.

Initially the bismuth subnitrate particles have a high positive zeta potential as shown at the left side of the diagram. The addition of monobasic potassium phosphate, the flocculating agent, reduces the charge and causes the zeta potential to fall to a point where the system is observed under the microscope to exhibit maximum flocculation. Sedimentation studies on the bismuth subnitrate suspension show that the H_u/H_0 is low initially, a condition that would lead to close packing and caking of the particles when they settle. Partial flocculation by potassium phosphate increases the volume of the sediment and the H_u/H_0 ratio reaches a maximum value in the noncaking zone of the diagram. A sulfamerazine suspension treated in this manner with aluminum chloride in our laboratory did not cake over a period of observation of about four years.

We observe in the diagram that addition of flocculating agent causes continued electrical discharge of the particles (a decrease in ζ potential) and finally a reversal of charge from positive to negative.

Addition of excess flocculent beyond the noncaking region in the diagram may cause a decrease in the H_u/H_0 ratio and a tendency of the suspension to cake again. From a diagram such as this we see that both sedimentation height and electrokinetic measurements can yield caking indexes for suspensions. Moreover, we learn from such a diagram that we should be able to prevent caking of suspensions by the controlled flocculation of the particles using agents bearing electrical charges of the opposite sign to that of the particles.

Application of Theory to Practice.—When we attempt to apply the above principles to the design of pharmaceutical suspensions we encounter one serious difficulty. Although we can prepare a highly flocculated system which does not cake, the particles settle rapidly and leave a supernatant layer even when an excess of flocculating agent is added, as seen in Fig. 7. This is considered undesirable in marketed products. Consequently, a suspending agent such as carboxymethylcellulose, bentonite, tragacanth, or a combination of these is added to produce a final product with a more uniform appearance. Most suspending agents belong to the class of negatively charged hydrophilic colloids and, when added to suspensions containing triply positively charged aluminum ions or other cationic flocculating ions, tend to form an unsightly stringy mass which rapidly settles and shows little or no suspending action.

Fig. 7.—Flocculation of a 15% suspension of sulfamerazine. 1 is deflocculated; 2, 3, 4, and 5 contain 1×10^{-3}, 2×10^{-3}, 3×10^{-3}, and 4×10^{-3} M Al^{+++}, respectively. From Bernard Haines, Ph.D. thesis, Purdue University, 1960.

While phosphate ions and other negatively charged flocculating agents result in no such incompatibility with the commonly used suspending agents, they obviously cannot be used to flocculate negatively charged particles such as the sulfonamides.

This difficulty may be overcome by coating the negatively charged particles with a monolayer of a positively charged coating agent such as a fatty acid amine or gelatin below its isoelectric point. Then the phosphate ion and other anionic flocculents can bring about controlled flocculation of the particles while remaining compatible with the suspending agent.

In 1945, Blythe patented a process in which a soluble sodium sulfonamide was precipitated from acid solution in the presence of gelatin. The powder, Microform sulfathiazole, which is prepared by this process, flows freely and does not cake in suspension. Dr. Blythe kindly supplied us with some of this powder for use in our studies. We found that although sulfathiazole itself is negatively charged, Microform sulfathiazole is positively charged, presumably due to the fact that the adsorbed gelatin in this product is on the acid side of its isoelectric point. In conformity with the principles that we have proposed, we attribute the freedom from caking of Microform sulfathiazole preparations to the positively charged and partially flocculated particles in suspension.

Consequently, we might reason that if fatty acid amines and gelatin on the acid side of its isoelectric point can be used to coat particles in suspension, other positively charged agents should also be effective in producing free-flowing powders with anticaking characteristics. We have done only preliminary tests with quaternary amines and have found that the principle also applies with these agents.

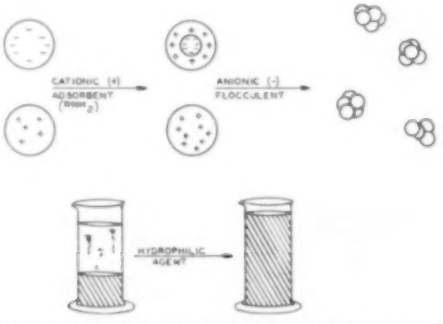

Fig. 8.—Sequence of steps in preparing a stable suspension.

Fig. 9.—1, Caked suspension of sulfamerazine, and 2, the same product properly prepared by the method of controlled flocculation. From Bernard Haines, Ph.D. thesis, Purdue University, 1960.

The technique of adsorbing a positively charged agent on suspension particles, followed by controlled flocculation with a negative ion, and finally stabilized with a negatively charged suspending agent is illustrated in Fig. 8. The scheme, as shown, should require no further comment; however, it is well to summarize the steps in this process as follows: (*a*) coat the particles with a positively charged agent which, obviously, must be checked for lack of toxicity before use; (*b*) add flavoring, coloring, and other ingredients in the ordinary manner; (*c*) partially flocculate the particles with a negatively charged agent if the zeta potential is not in the proper range for freedom from caking; (*d*) finally, add a minimum amount of the desired suspending agent or mixture of suspending agents and again check the zeta potential to assure optimum conditions of noncaking.

On the right hand side of Fig. 9 we see the kind of product that can be prepared by the application of these principles. In contrast on the left we see a suspension that was prepared by the use of deflocculated particles and a viscous suspending agent. The sediment which can be seen in the bottom of the container on the left cannot be redispersed, even by vigorous agitation of the bottle. The product on the right contains just sufficient suspending agent to suspend the small flocs and allow rapid redistribution of dosage on agitation. The method suggested here should be applicable for negatively charged, positively charged, or neutral particles. Any one of these types or a mixture of powder types may be coated with the positively charged agent and treated as outlined above to produce stable, noncaked suspensions.

When this method is followed it should be necessary to use only a minimum of suspending agent to provide a uniform product of fine, well dispersed floccules and allow easy redispersion of any sediment by simple inversion of the container. A suspension having a high consistency cannot yield uniform dosage, does not pour well from the container, and may leave a film in the mouth, leading to an unpleasant aftertaste. Thixotropic suspending agents which do not flow freely when agitated or which set up too rapidly after they are disturbed show these disadvantages. Therefore, it is suggested that the principle of controlled flocculation, coupled with the use of small quantities of carefully chosen suspending agents, be employed in the preparation of pharmaceutical suspensions.

It must be added that additional work should be done by those who wish to apply this method in the design and manufacture of suspensions. New coatings must be tested for freedom from toxicity, and the effect of the coating on the rate of release of the active medicament must be studied.

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An Industrial Approach to Suspension Formulation

By JOSEPH C. SAMYN

The current status of suspension testing has been reviewed while the role of a Brookfield viscometer as an empirical instrument yielding valuable comparative data has been emphasized. The hydration characteristics of the pharmaceutical clays have been considered and the importance of hydration in proper clay utilization and in scale-up has been pointed out. The combined use of a pseudoplastic and plastic suspending agent has been advocated because of the desirable properties such a system possesses. Finally, a formulation design has been proposed whereby the flow properties necessary for a satisfactory suspension are determined in a series of simple preparations.

THERE ARE MANY problems associated with the formulation of an acceptable suspension. The proper pH, preservative, color, and flavor, as well as the study of the chemical stability of the product, must each be determined. These problems are not, however, unique to the suspension-type product, but are more or less common problems of all oral liquid dosage forms.

One of the tasks of suspension formulation centers around the sedimentation characteristics of the insoluble phase that is present. There are two approaches to the formulation of an insoluble material in a liquid preparation: (*a*) The insoluble powder can be allowed to settle in the vehicle provided that it can be resuspended before dosage removal. An excellent review of the factors involved in this approach was recently presented by Jack (1). Usually no attempt is made in this method to disguise the two-phase system. (*b*) The powder may be maintained in suspension with a minimum amount of separation. This latter technique, where feasible, can be expected to yield a more pharmaceutically elegant preparation. Each of these approaches has merit and the requirements of each individual

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laboratory would probably dictate the method selected.

This paper deals with the second approach; namely, attempting to maintain the insoluble powder in uniform suspension indefinitely. We wish to deal first with the factors involved in sedimentation and then review briefly some basic concepts of viscosity.

PRELIMINARY CONSIDERATIONS

Theoretically, the rate of settling of an insoluble material in dilute suspension is usually discussed in terms of Stoke's law. Higuchi (2) has recently applied the Kozeny equation to concentrated suspensions. For our purposes, a qualitative look at Stoke's equation will reveal the effect of the variables on the rate of sedimentation, although Stoke's law itself does not directly apply to pharmaceutical suspensions.

$$V = K(P_p - P_s)d^2/\eta$$

From this equation, in which the symbols have their usual meanings, it can be seen that the rate of settling will approach zero as the density difference between particle and vehicle approaches zero, as the particle size is reduced, and as viscosity is increased to infinity. For materials of low density ranging from 1.1 to 1.3, adjusting the vehicle density to equal the powder density will minimize suspension difficulties. Two recent patents were issued employing this principle (3, 4). Unless the particle size is reduced to colloidal dimensions, however, sedimentation will not be prevented by size reduction alone.

It is the viscosity term in the above equation that is of particular interest at this time. While pure liquids have a definite viscosity, a suspension can be expected to have many viscosities, depending on the treatment imposed. In order to clarify this statement, consider briefly the different flow properties of the various rheological systems.

Figure 1 is a typical plot of the various flow properties that are of interest in pharmaceutical liquid formulations. The ratio of shearing stress to rate of shear is defined as the coefficient of viscosity. In curve 1, this ratio, the Newtonian viscosity, is a constant. Curve 2, obtained with pseudoplastic materials, shows the ratio of shearing stress to rate of shear to be continually changing, that is, the viscosity is continually decreasing with increasing shear rate. Plastic materials, curve 3, exhibit a finite shearing stress called a "yield value" above which the ratio of shearing stress to rate of shear is a constant, independent of shear rate.

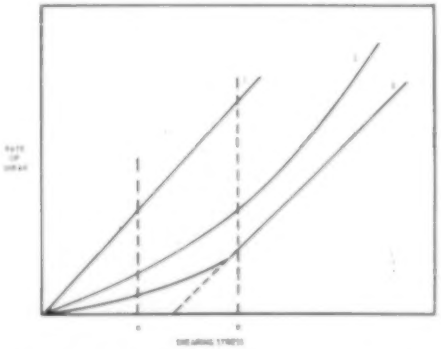

Fig. 1.—Typical plot of the various flow properties in pharmaceutical liquid formulations.

There are two processes involving different shearing stresses that concern us in suspensions: the low stress caused by the sedimentation of a particle and the higher stress induced by shaking or pouring the preparation. Consider the shearing stress induced by a particle as it falls through a vehicle; one would expect it to be low. Further, with a small particle whose density differs only slightly from the media and in a media that is moderately viscous, one would expect this shearing stress to be even lower. In Fig. 1, point *A* represents this shearing stress and point *B* corresponds to the higher shearing stress caused by pouring the preparation. For the Newtonian curve, curve 1, the viscosity is the same at points *A* and *B*. In order to prevent settling in such a system, the viscosity of the vehicle would have to be excessively high and the product would be unpourable. For the pseudoplastic flow curve, curve 2, the viscosity at point *A* is greater than at point *B*. This means that at the low stresses induced by a falling particle, the effective viscosity of a pseudoplastic system is much greater than the effective viscosity under pouring conditions. From this viewpoint, it can readily be seen that a pseudoplastic vehicle is a better choice for a suspending agent than a Newtonian vehicle.

Consider now the case of the plastic suspending agent, curve 3. In this instance, at point *A* the shearing stress produced by a settling particle is less than the yield value of the suspension and no flow or sedimentation should occur. At point *B*, however, the yield value is exceeded and the preparation flows with a viscosity equal to the reciprocal of the slope at point *B*. A suspension with this flow curve should not settle, yet it should remain pourable. The essential feature of this hypothetical case is the position of the yield value of the suspension between the points *A* and *B*. If the yield value were higher than point *B*, the suspension would show poor pouring properties. If it were below *A*, there would be sedimentation. Further, since point *A* can be reduced to a lower shearing stress by employing small particles, minimizing density differences and having a viscous pseudoplastic media, it then becomes easier to obtain a yield value between *A* and *B*. In actual practice, then, the combined use of a pseudoplastic and a plastic suspending agent would appear to be an excellent media for suspending an insoluble material.

THE SUSPENDING SYSTEM

There are many factors which determine the best pseudoplastic and plastic suspending agent for a particular preparation. Incompatibilities, ease of preparation, availability of pure uniform material, and the pH, and the temperature stability of the material should each be considered. Several publications have reviewed these factors and analyzed the existing pseudoplastic suspending agents in light of these properties (5–9). Since the synthetic pseudoplastic agents, in general, can be expected to show less variation than the naturally occurring materials, they are probably to be pre-

ferred. Sodium carboxymethylcellulose is an excellent choice provided interactions with cationic materials, as reported by Higuchi and Kennon (10), are not a problem. Methylcellulose is widely used. Sodium alginate is one of the better materials obtained from a natural source.

In the past, the main source of plastic flow properties for aqueous suspension has been bentonite. Presently, several other commercial clays have become available and they are preferable to bentonite because of their whiter color and greater uniformity, among these are Veegum H. V.,[1] Thix,[2] and Macaloid.[3] The Carbopols[4] have been reported to possess a yield value and show plastic flow properties (11), but because of their low tolerance to electrolytes, usually present in fairly high concentration in pharmaceutical suspensions, the Carbopols appear to be of limited value.

Therefore, to obtain the desired flow properties, one of the pseudoplastic suspending agents and one of the clays should be used in combination. Sodium carboxymethylcellulose and Veegum H. V. have been used satisfactorily and will be selected for this discussion.

IMPORTANT PROPERTIES OF THE SUSPENDING AGENTS

For the pseudoplastic materials, sodium carboxymethylcellulose, methylcellulose, and so forth, the literature has been adequate and very little difficulty has been encountered in their use. On the other hand, the clays have been difficult to use pharmaceutically, in spite of an extensive literature and excellent books on the subject by Grim (12) and Marshall (13). The mechanisms for the behavior of clays in water and the influence of salts and surfactants are as follows (13): clay particles added to water swell and sorb water in the expandable c-spacing of the crystal lattice. The driving force for this sorption is the mutual repulsion of negative charges carried by the clays. The addition of salt to the clay slurry neutralizes this negative charge and flocculates the system. This results in a large viscosity increase. In addition to this viscosity rise, the salt addition prevents further sorption of water since the driving force, the repulsion of like charges, has been neutralized. Surfactants are believed to exert their influence on clays by increasing their hydrophilic nature, i. e., increasing the sorbed water sheath on the particles. This causes the clay particles to act like larger units and gives rise to increased viscosity.

The properties of the clays studied in our laboratories may be conveniently divided into two categories: (*a*) the clay concentration required and its interactions with various additives, and (*b*) the hydration characteristics of the clays.

Clay Concentrations and Salt Effects.—Most of the previous studies (14–19) using the clays in pharmaceutical suspensions had concentrations in the 2–5% range. Few, if any, salts or surfactants were included in these studies and high concentrations of the clays were necessary to develop sufficient viscosity. In a commercial suspension, large concentrations of salts and surfactants are normally added to a suspension. It is proposed that a concentration of a clay of 1% or less in the final product develops ample viscosity due to the flocculating effect of the added salts and surfactants. This effect can be seen for a 1% Veegum H. V. slurry in Fig. 2. It is evident that the consistency obtained with a clay is dependent on the relative concentrations of clay, salts, and surfactants.

Hydration Characteristics.—When studies of a basic nature are performed on clay materials, the clays to be studied are initially dispersed in water for long durations to insure complete hydration. Hydration periods as long as several months have been reported in the literature. Even the U. S. P. (20) swelling test for bentonite is carried out for twenty-four hours to insure "complete hydration." However, when a clay is used in a commercial suspension, incomplete hydration is apt to be the rule. It is usually preferred to manufacture the entire product from start to finish in a single tank in as short a time as possible. Therefore, the hydration may be continued only until the slurry appears to be "smooth and milky," possibly only an hour.

To demonstrate the importance of the degree of hydration, Tables I and II were prepared from data on two commercial suspensions containing Veegum H. V. where the degree of hydration of the clay was intentionally varied.

In Table I, the acceptable consistency range is 150–250 Brookfield units. The 780 figure is markedly thicker than the 220 Brookfield viscosity product. The only change in these suspensions is the extent of hydration of the 0.6% Veegum H. V. in each of the formulas.

In Table II, the importance of the clay hydration on the viscosity of a second formula is shown.

Having established the importance of the state of

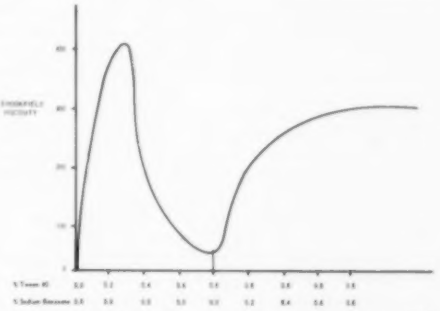

Fig. 2.—Influence of a salt and a surfactant on the viscosity of a clay.

TABLE I.—INFLUENCE OF EXTENT OF HYDRATION ON VISCOSITY IN FORMULA A

Hydration State	Brookfield Viscosity[a]
Fully hydrated	780
Normal production hydration	220
No hydration	95

[a] Readings taken on the No. 2 spindle at 30 r. p. m. after three minutes. These readings were then multiplied by the factor 10.

[1] Product of R. T. Vanderbilt Co., 230 Park Avenue, New York 17, N. Y.
[2] Product of National Lead Co., 111 Broadway, New York 6, N. Y.
[3] Product of The Inerto Co., 1489 Folsom St., San Francisco 3, Calif.
[4] Product of B. F. Goodrich Chemical Co., 3135 Euclid Ave., Cleveland 15, Ohio.

Table II.—Effect of Clay Hydration on Viscosity of Formula B

Hydration Time, min.	Brookfield Viscosity*
Complete—∞ time	3,200
30	2,000
10	1,600
5	1,100
0	400

* Readings taken on the No. 3 spindle at 30 r. p. m. after four minutes of rotation. These readings were then multiplied by the factor 40.

Table III.—Inhibitory Effect of Salts Present During Hydration of Veegum H. V.

Hydration Medium	Brookfield Viscosity*
Distilled water	370
Tap water	200
0.01% Calcium chloride solution	220
0.5% Sodium benzoate solution	28

* Readings taken on the No. 2 spindle at 30 r. p. m. after three minutes. These readings then were multiplied by the factor 10.

Table IV.—Effect of Concentration on the Hydrating Slurry

Hydration Time, min.	Brookfield Viscosities*	
	2% Sample	4% Sample
2	60	60
5	90	80
10	110	90
20	150	110
40	180	120
80	210	130

* Readings taken on the No. 2 spindle at 30 r. p. m. after three minutes. These readings were then multiplied by the factor 10.

hydration as regards product viscosity, consider some of the factors which affect the degree of hydration of a pharmaceutical clay. Three factors will be mentioned here: (*a*) the media used for the hydration; (*b*) the concentration of the clay in the hydrating slurry; (*c*) the mixing intensity and duration.

The Hydrating Media.—In Table III, the inhibiting effects of salts present during the hydration of the clay are illustrated. The data in Table III were obtained by hydrating 1% Veegum H. V. in each of the specified media for one-half hour on a laboratory shaker and then adding 0.5% sodium benzoate. It will be noted that when the hydration is carried out in the sodium benzoate solution, there is essentially no viscosity increase. This entry was included in the table to illustrate that the effective hydration time ends with the addition of the salts to the clay-water slurry.

Concentration of the Clay in the Hydrating Slurry.—Although the clay may constitute only about 1% of the final formula, its concentration in the hydrating slurry would conceivably be higher due to a limited amount of water available for this portion of the formulation. Since clays at higher concentrations develop viscosity, it is possible that the rate of hydration could be affected. Table IV illustrates the reduced viscosities obtained after different time intervals when Veegum H. V. is hydrated as a 4% slurry and as a 2% slurry. The samples were all diluted to 1% clay and 0.5% sodium benzoate was added to each before the viscosities were determined on a Brookfield viscometer in a standard manner. It will be noted that the 4% sample gave lower viscosities at each of the time intervals than did the 2% sample.

The Mixing Intensity and Mixing Time.—This factor is of particular importance in the scale-up process since widely different mixing conditions usually prevail between a laboratory and a production operation. In the laboratory a high-speed mixer or a shaker is apt to be employed. In production, the mixing assembly often consists of a large tank with an impeller of large diameter, driven at low speed. Working with such different conditions, the extent of hydration of the clay is not duplicated from laboratory to production simply by hydrating for the same time period. Rather, in one instance, an hour of production hydration was necessary to duplicate the viscosity obtained with only fifteen minutes of laboratory stirring. If time and conditions are adjusted so that a clay is hydrated to the same extent in production as it is in the laboratory, it will make the same contribution to the final consistency of the product.

Another study carried out under controlled mixing conditions measured the variations in hydration rates of different lots of Veegum H. V. Table V tabulates hydration data for different lots of Veegum H. V. obtained with the following standard hydration procedure: A standard mixing assembly (fixed container, agitator, speed) with fixed concentrations of clay and distilled water are used. A routine procedure of hydrating the slurry for predetermined times, sampling, and adding a fixed amount of salt to the samples to quench and develop the viscosity, is employed.

Four different lots of Veegum are listed in Table V. These particular lots were selected to show the variation in hydration rates observed in our laboratories. Replicate samples are shown for three of the four lots to illustrate the reproducibility of the hydration test and the Brookfield viscosity measurements. For any hydration time, the viscosities developed for the various lots of clay can be quite different. Therefore, in using these different lots of clays in a suspension, the quantity of clay or time of hydration must be adjusted to maintain uniform product viscosity.

PRESENT STATUS OF TESTING AND CONTROL OF SUSPENSIONS

Viscosity measurements are usually made with either a single point instrument such as a Brookfield, or with a high shear viscometer capable of yielding the entire flow curve. There are several literature references (11, 21–23) suggesting the use of the Brookfield viscometer with methods for converting data to the basic terms of viscosity, plastic viscosity, yield value, etc. For suspensions that are known to be plastic, this may be possible. Normally when dealing with suspensions, however, Brookfield data should be treated as comparative empirical data. The Brookfield measurements cited in this paper were performed in a standard manner on samples of similar composition. The resulting data has been shown to be reproducible and of value.

TABLE V.—CLAY HYDRATION STUDIES[a]

Hydration Time, min.	Brookfield Viscosity[b] Lot 558774		Lot 561336		Lot 567601		Lot 562619
2	80	80	60	60	50	50	30
5	120	100	90	80	50	60	40
10	160	120	110	110	70	70	50
40	230	240	180	190	100	100	130
160	280	280	220	...	130	...	180

[a] 1% Veegum H. V., 0.5% sodium benzoate.

[b] Readings taken on the No. 2 spindle at 30 r. p. m. after three minutes. These readings were then multiplied by the factor 10.

With the more elaborate high shear viscometers, the full flow curve may be measured. For the Newtonian and plastic systems, the results may be expressed in terms of viscosity or plastic viscosity and yield value. Vincent, *et al.* (24), successfully employed a Hercules Hi-Shear viscometer to measure the yield values of high solid penicillin suspensions. Equally good experimental data can be obtained for pseudoplastic materials. It is often difficult to present the flow curve in a meaningful manner because of the limited basic relationships presently available.

It is felt that the Brookfield or other similar instrument is useful as an empirical measure of the relative consistencies of similar preparations. The information obtained with such an instrument should aid in the formulation and in the routine control of suspensions. However, it would appear that any basic advance in the application of rheology to suspension formulation will come about through the use of a high shear viscometer with appropriate theory.

Sedimentation studies should also be performed on pharmaceutical suspensions. A variety of methods of measurement have been used, such as: visual observation of the clear supernatant, the use of a sedimentation balance, sample removal at a fixed height as a function of time, etc. The use of elevated temperatures and centrifugation to accelerate room standing have been largely unsuccessful. At present, room standing appears to be the only reliable measure of sedimentation.

Any attempt to maintain an insoluble powder in permanent suspension will be met with varying degrees of success. Shaking procedures with subsequent sampling for uniformity would be adequate means of testing for resuspendibility.

Other more qualitative attributes of suspensions such as pourability, the flow pattern on the inside of the container, the amount and ease of removal of entrapped air, etc., are of importance in particular instances. The behavior of a product under prolonged shear or elevated temperatures is often helpful in understanding the changes that occur in suspensions upon aging, or those which might occur in production manufacture with its inevitable deviations from specified instructions.

PROPOSED METHOD OF FORMULATION

Normally, the desired consistency for a given suspension is arrived at in the presence of all the ingredients in the complete formula. While many of these ingredients are added to perform a specific function, such as to preserve or buffer, they unfortunately often affect the suspending agents used in these suspensions. In particular, the various salts that are added tend to flocculate the clays that are used. This flocculating action and the concentrations of the suspending agents largely determine the consistency of the product. Therefore, consider a plan whereby the problem of suspending an insoluble powder would be isolated from the other studies necessary for the formulation of a satisfactory suspension.

It would appear that the consistency necessary for adequate suspension of an insoluble material could be determined in a simple system containing the powder to be suspended, the suspending agents, and a representative salt. If the flow properties of the most satisfactory simple preparation were then measured, it would be conceivable that an adjustment in the concentrations of the suspending agents, to account for the effect of the other additives, could be made so that these approximate flow properties would be duplicated in the complete formula. A method of formulation such as this would enable one to establish the necessary flow properties independent of a knowledge of the other ingredients. It would allow one to study the suspension aspects of the formula concurrently with the other studies. This procedure is based on two premises: (*a*) The flow properties found to adequately suspend the powder in the simple system should also be suitable for the complete formula, provided the particle size distribution and degree of dispersion are essentially not affected by the other ingredients added; and (*b*) the major difference between the simple and complete formula is an adjustment in the clay concentration to account for the various salts that are added. A formulation plan of this type might well be divided into three parts: (*a*) the particle to be suspended; (*b*) the preliminary formulations; (*c*) the final formulation.

The Particle to be Suspended.—Particle characteristics such as particle size, shape, and size distribution, should be determined. For the low solids suspensions, less than 10%, microscopic examination is probably adequate. For the higher concentrations, quality control tests based on suitable particle size data are desirable. Modifications of the particle size characteristics by milling or recrystallizing should also be undertaken if the material is unsuitable for suspension formulation.

The minimum amount of a suitable dispersant should be employed and the approximate apparent density of the powder with this dispersant should be measured. It is felt that a powder in at least a semidispersed state gives a better gross appearance and feel to a product than does a flocculated preparation. In addition, an earlier premise of this paper was to reduce the particle settling rate by employing small particles, settling independently.

Preliminary Formulations.—These formulations should contain the desired concentration of the powder to be suspended, a suitable dispersant if necessary, the pseudoplastic and plastic suspending agents, a typical salt, and possibly sucrose and glycerin if these materials are expected to appear in relatively high concentrations in the final formulation. A series of these preliminary suspensions should be formulated with the pseudoplastic and plastic suspending agents at several different concentrations. A possible design consisting of 16 preparations with Veegum H. V. and sodium car-

hoxymethylcellulose in 0.3% increments from 0–1.2% has been used.

There are several features of such a design which should be considered: (*a*) The amount of work required to prepare these sixteen suspensions. Each suspension in this series need not be formulated independently. On the contrary, the proper concentrations of suspending agents for each suspension may be obtained by taking the necessary volumes of previously prepared concentrates of these agents. In addition, a fixed volume of a stock slurry of the insoluble powder, dispersant, salt, sucrose, and glycerin can be added to each suspension. All suspensions are then brought to the required volumes.

(*b*) The range of consistencies covered in this type of a plan. It is felt that with these concentrations of the suspending agents, the suspensions prepared will cover the range of consistencies that could possibly yield a satisfactory pharmaceutical suspension. From this series, one or several of these formulas can be expected to show satisfactory suspension properties. Although the remaining samples will be unacceptable for one reason or another, they still perform several important functions: While judging the quality of an isolated suspension is often a difficult task, the selection of the most satisfactory of a series of similar suspensions should be less difficult because of the comparisons available. By covering the entire range of flow properties possible, one can observe the degree of latitude available for the formulation of a satisfactory product. Since the formula, which at first appeared to be the most satisfactory may in time show some undesired property, the remaining suspensions in the series will permit further comparisons without further aging.

(*c*) The elimination of the samples with only one of the suspending agents. It is felt from our experiences with these systems that suspensions relying totally on sodium carboxymethylcellulose will, in time, settle. On the other hand, suspensions containing only the clay will entrap air and present pouring difficulties.

(*d*) The relatively large increments in the suspending agent concentrations. It is felt that with additional experience, the preliminary powder measurements should make it possible to select a narrower range of concentrations which could then be covered in smaller increments. For example, if the particles are less than 10 μ and have a density less than 1.1 the lower concentration range of suspending agents should be satisfactory.

(*e*) The method of measurement of the consistency of the suspensions. Although the Brookfield viscometer might possibly be employed, we have felt that the flow properties of these suspensions would probably be more adequately described with a flow curve from a high shear viscometer. Therefore, a modified Hercules Hi-Shear viscometer was used to obtain a semiequilibrium downcurve. The second or third downcurve run with this instrument appeared fairly stationary and was therefore selected. When it is realized that the duplication of this downcurve in the final formulation is only approximate and that there must be considerable latitude in the acceptable flow properties of a suspension that will be repeatedly manufactured, the selection of this downcurve appears justified.

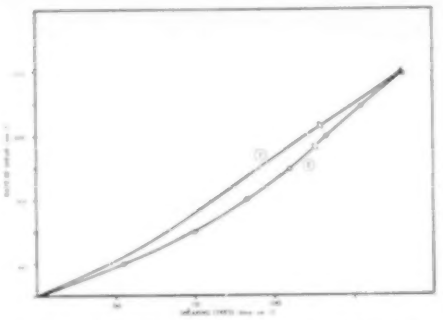

Fig. 3.—Typical down curves for a preliminary and a complete suspension.

The Complete Suspension.—After the concentrations of the other ingredients in the formulation have been established, the concentrations of the suspending agents are adjusted so that the complete formulation will possess flow properties in the same range as those found to be acceptable in the preliminary suspensions. Figure 3 illustrates the downcurves obtained for the most acceptable preliminary suspension, curve 1, and the corresponding final formulation, curve 2. The preliminary formula contained 0.3% Veegum H. V. and 1.2% sodium carboxymethylcellulose as the suspending system. In the final formulation, these concentrations were adjusted to 0.6% Veegum H. V. and 1.3% sodium carboxymethylcellulose to obtain the flow curve shown. The downcurves for these preparations can be seen to be reasonably close together and, for the reasons stated earlier, this degree of duplication of the flow properties appears satisfactory for the present time.

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Mixing of Solid-Liquid Suspensions

By J. Y. OLDSHUE

The physical principles relating to the mixing of solid-liquid suspensions are applied to the dispersion of solids in liquid by different types of equipment.

PHARMACEUTICAL SUSPENSIONS usually involve mixing. Mixing is commonly carried out by a rotating impeller which imparts a fluid velocity in the mixing vessel. The term "mixing" involves many different operations. To orient the entire field, Table I illustrates the basic mixing classifications into fluid phases and then into the two basic classifications of mass transfer or physical dispersion. In mass transfer processes, the material is actually being transferred from one phase to the other, while in physical dispersion we are primarily trying to suspend one phase in another, or blend similar materials.

This paper is concerned entirely with the suspension of solids in liquids.

A careful distinction should also be made between the horsepower required for a process performance which is set by the process requirements, and the relationship between power, speed, and impeller diameter, which is a physical relationship existing for any particular impeller type.

The topics to be treated are: the principles of mixing, the physical relationship between power, speed, and impeller diameter, and a distinction of process requirements.

TABLE I.—MIXING PROCESSES

Mass Transfer		Physical Dispersion
Extraction	Liquid-liquid	Emulsions
Dissolving, leaching	Solid-liquid	Suspension
Absorption	Gas-liquid	Foams
Heat transfer	Heat transfer	Blending
	Blending	

MIXING PRINCIPLES

Mixing Impellers.—Mixing impellers are divided into two basic classifications, radial flow and axial flow. Axial flow impellers are characterized by propellers, Fig. 1, and fan turbines, Fig. 2. Radial flow is characterized by turbines, Fig. 3, and paddles, Fig. 4.

In tanks that are unbaffled, Figs. 5 and 6, the flow patterns from all impellers are somewhat similar. In tanks that are baffled, the flow patterns are quite distinct between axial and radial flow, Figs. 7 and 8.

There are many other variations of impeller design from those illustrated in the figures. However, their characteristics mixing-wise can be included in the two broad classifications, axial flow or radial flow. The following discussion will amplify some of these mixing principles.

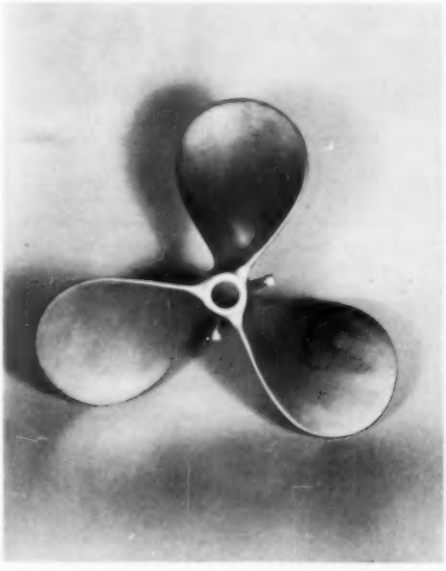

Fig. 1.—Typical marine type propeller.

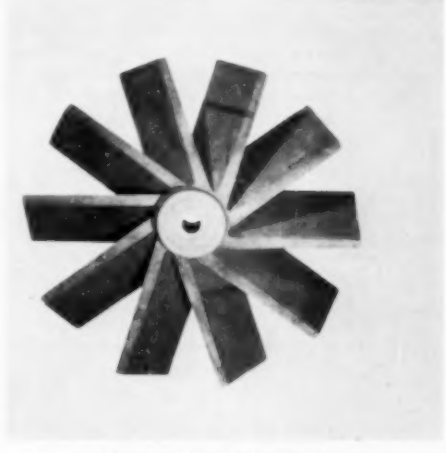

Fig. 2.—Typical fan turbine.

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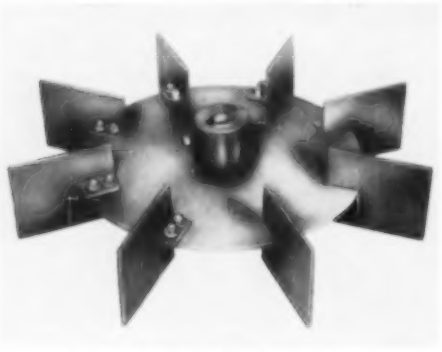

Fig. 3.—Typical flat blade turbine.

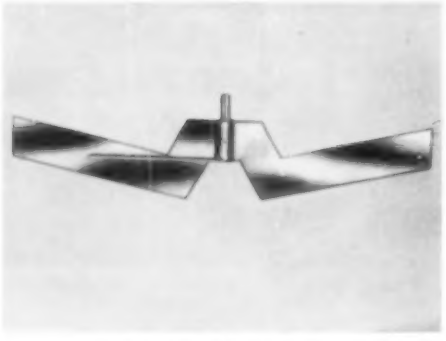

Fig. 4.—Typical paddle.

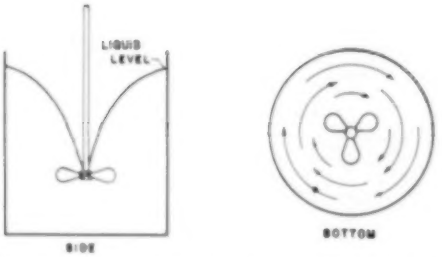

Fig. 5.—Flow pattern, propeller on center in unbaffled tank.

Baffles.—Tanks may be classified as baffled or unbaffled in terms of their general fluid characteristics. A baffled tank has the following characteristics: (*a*) top-to-bottom turnover with complete intermixing of all fluid streams throughout the entire vessel, (*b*) the absence of a vortex through which air is drawn down into the fluid, (*c*) top-to-bottom turnover as contrasted to a swirling flow pattern.

In water-like materials, baffles are commonly used. These are normally four in number, each one-twelfth of the tank diameter in width. Figure 7 illustrates a propeller operating under baffled conditions in low viscosity fluids.

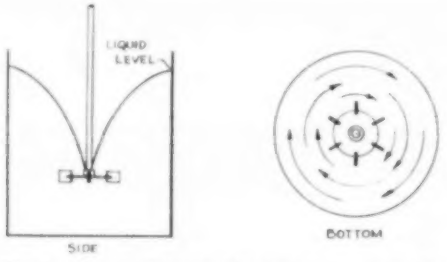

Fig. 6.—Flow pattern, turbine impeller on center in unbaffled tank.

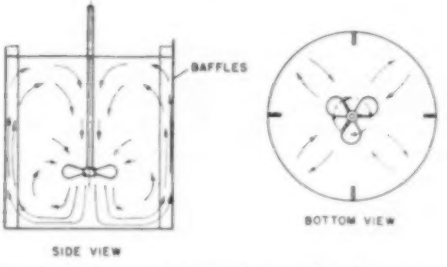

Fig. 7.—Flow pattern, propeller positioned on center with baffles.

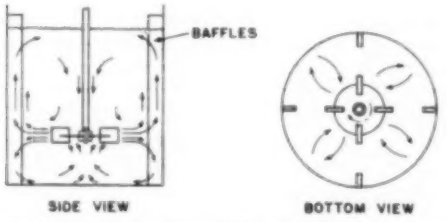

Fig. 8.—Flow pattern, turbine positioned on center with baffles.

In addition, it is possible with a propeller to use an angular off-center position which sets up a top-to-bottom flow pattern essentially equivalent to that in a baffled tank; Fig. 9 illustrates this condition.

In some cases an unbaffled flow pattern is used to suck down powders from the surface or to entrain air.

In low viscosity fluids, unbaffled operation can set up an unstable flow pattern which gives erratic results in scale-up. In addition, it causes severe fluid forces on the mixing impeller and causes an unbalanced load on the mixer drive.

In more viscous materials, 1,000 cps. and up, it is sometimes possible to achieve a top-to-bottom flow pattern without the use of baffles. However, the amount of power that can be used without swirling or vortexing is limited. Since the flow pattern depends upon the ratio of surface area to volume, the scale-up characteristics of unbaffled tanks are not as predictable as those of a baffled system.

For solid suspension, baffles should be set off the

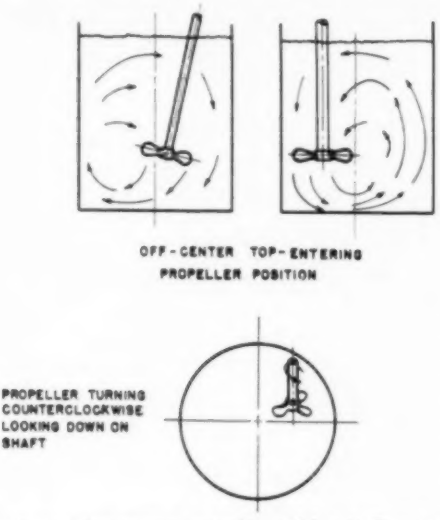

Fig. 9.—Flow pattern, propeller positioned off-center without baffles.

wall 2 to 4 inches to prevent solids accumulation at the wall.

Fluid Flow and Fluid Shear.—One of the most useful concepts in evaluating mixer performance is that of impeller pumping capacity and fluid shear. The term "fluid regime" is used to identify the condition of power input to the system and the distribution of that power input into flow and fluid shear. The power consumed by the mixing impeller produces flow and an impeller head

$$P = QH \qquad \text{(Eq. 1)}$$

The flow produced by the impeller, Q, is measured as pounds of fluid flowing per hour.

Studies on the pumping capacity of mixing impellers show that for a geometric series of impellers, the flow is given by the proportionality

$$Q \propto ND^3 \qquad \text{(Eq. 2)}$$

The impeller head, H, is an important variable since it is related to the fluid shear which exists in a fluid stream as it leaves the impeller blades. This tends to set up turbulence at the boundary of the fluid stream.

A detailed discussion of fluid shear is not included here, but it is related to the ability of a mixing system to disperse agglomerates and cause mixing in small elements of fluid.

We are normally interested in the ratio of flow to fluid shear at a given level of power. The flow to fluid shear ratio, or flow to head ratio, is related to the impeller size, D, and tank size, T, by the equation

$$[Q/H]_p \propto [D/T]^{3/2} \qquad \text{(Eq. 3)}$$

A large impeller must run at slower speed than does a small impeller at the same horsepower. A large impeller running at slow speed gives a large pumping action and a low level of fluid shear, while a small impeller at high speed gives a high level of fluid shear and a low level of fluid flow.

In the solid suspension process, pumping action is of prime interest since this distributes the fluid throughout the entire system. Fluid shear may or may not be of interest depending upon whether solid dispersion or break up of agglomerates is required.

Additional details of flow to fluid shear ratios are given in the article by Rushton and Oldshue (1).

IMPELLER POWER CONSUMPTION

If the mixing process is analyzed in terms of the power level, then the speed of the impeller is related to its design and the fluid properties.

The power consumption of a mixing impeller is related to its speed and diameter and position in the tank. The power consumption is correlated by means of dimensionless groups shown in Fig. 10. The power number is the power divided by the specific gravity, cube of the impeller speed, and the 5th power of the diameter, $Pg/\rho N^3D^5$. This group is related to the Reynolds number which is given by the speed, square of the diameter, and fluid density divided by the viscosity, $ND^2\rho/\mu$.

It can be seen from Fig. 10 that there are three definite ranges. In the turbulent region, $N_{Re}>10^4$, power varies with the cube of the impeller speed and the fifth power of the diameter and directly with the density of the liquid

$$P \propto \rho N^3 D^5 \qquad \text{(Eq. 4)}$$

Therefore, changes in impeller diameter or speed should be made with caution since they have large effects on the power drawn by the mixer.

There is a wide range of viscosities under which the power consumption of a turbine type mixer is not affected by viscosity, Fig. 10. The power drawn by a turbine impeller will decrease and then return to the power drawn in water as viscosities go from 1 to about 10,000 cps. Care must be exercised, however, that the unit has sufficient power to do the mixing job in all of these viscosities.

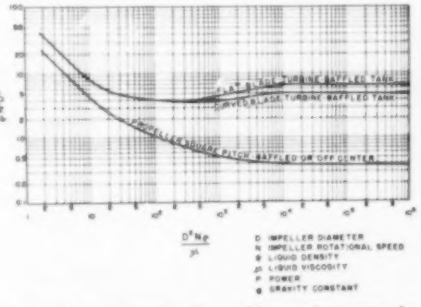

Fig. 10.—Power number Reynolds number curve for three different impeller types.

There is a greater affect of viscosity in propeller type units as shown by the continuous increase in the Reynolds number power number curve above Reynolds number = 10^4.

The power consumption of the propeller is affected by viscosity to a marked degree above about 500 cps.

In the transition area, $10 < N_{Re} < 10^4$, there is a variable exponent on speed and impeller diameter.

The viscous region, $N_{Re} < 10$, is not reached unless the viscosities are on the order of 20,000 cps. or higher. In this range, power varies with the square of the speed, cube of the diameter, and directly with viscosity. Specific gravity of the fluid has no effect

$$P \alpha \mu N^2 D^3 \qquad \text{(Eq. 5)}$$

PROPERTIES OF LIQUIDS AND SOLIDS

Liquid Phase.—The term "viscosity" has been used several times in the discussion so far. Viscosity is the ratio of shear stress and shear rate. Most measuring devices use a rotating impeller operating in the viscous range so that the shear stress and shear rate are related by the viscosity. The shear rate is related to the speed of the measuring device. The shear stress is measured by the torque in the measuring instrument and if there is a constant proportionality between torque and speed of the measuring instrument, the viscosity is constant at all shear rates, and we have a Newtonian material. This is illustrated in the curves in Figs. 11 and 12.

If the ratio of shear stress to shear rate decreases with increasing shear rate, we have the most common type of non-Newtonian behavior, that being pseudoplastic materials. It turns out that most pseudoplastic materials can be classified by a straight line relationship on a logarithmic plot, thus the plot shown in Fig. 12 is often used rather than the arithmetic plot used in Fig. 11.

Two other classes of fluid, dilatant and Bingham plastic, are also shown in Figs. 11 and 12.

The importance of non-Newtonian fluids depends upon the mixing application. If the shear rates are high enough and the apparent viscosity in such a range that we are in the turbulent or transition area,

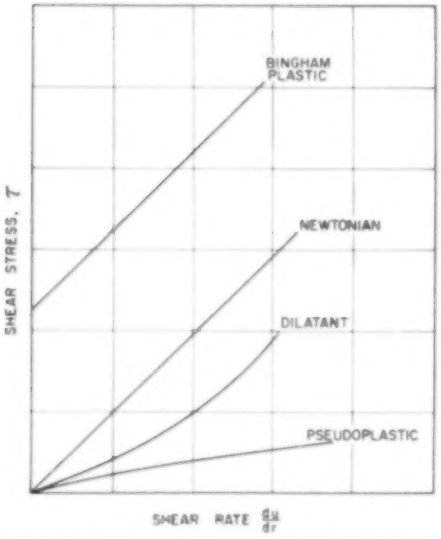

Fig. 11.—Shear stress *versus* shear rate for various types of fluids on an arithmetic plot.

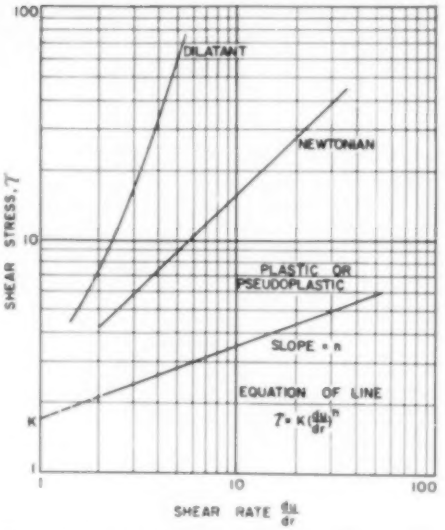

Fig. 12.—Shear stress and shear rate for various types of fluids on a logarithmic plot.

the non-Newtonian properties during the mixing process are often of minor importance. However, once the mixer is turned off, the pseudoplastic properties have a marked effect on the tendency of solids to separate out.

Most viscosimeters operate at shear rates much lower than those in a mixing tank. Data taken with these viscosimeters will give a good indication of the viscosity which is effective in maintaining a suspension after the mixer is turned off. However, to obtain the "apparent" viscosity of a material at the shear rates existing in a mixing tank, viscosity data should be obtained on a mixing system.

For pseudoplastic materials, data in the literature (2, 3) indicate that viscosity is related to impeller speed. In general, a plot of "apparent" viscosity *versus* impeller speed is useful for calculating the power consumption of the mixer under various conditions (Fig. 13).

A procedure which is used to determine the "apparent" viscosity of a fluid is to first prepare a curve of power number *versus* Reynolds number of a Newtonian liquid in the viscous range, shown in Fig. 14. Then, runs are made on the non-Newtonian test liquid on a dynamometer equipped to measure horsepower. At any given speed, the power number can be computed and the "apparent" viscosity can be computed from the curve shown in Fig. 14.

Solid Phase. In terms of the solid phase, one of the most important characteristics is the settling velocity of the solids. We may make a distinction between two classes of suspension. If the particles have an essentially zero settling velocity, for all practical purposes a permanent suspension is maintained. If the particles have a finite settling velocity, the solids will eventually fall out of suspension after mixing. Settling velocities above 1 foot per minute mean that the solids will fall rapidly out of suspension, while velocities below 1 foot per second

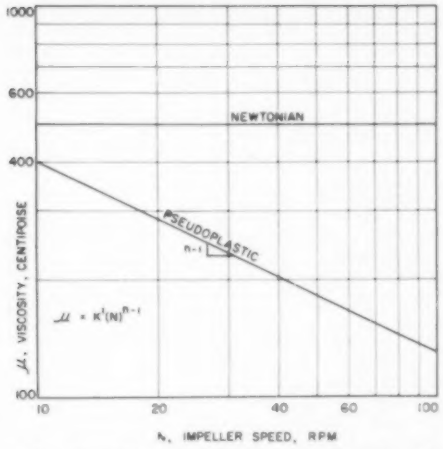

Fig. 13.—Viscosity of fluid at various impeller speeds. μ is viscosity, n-1 is the slope of the curve.

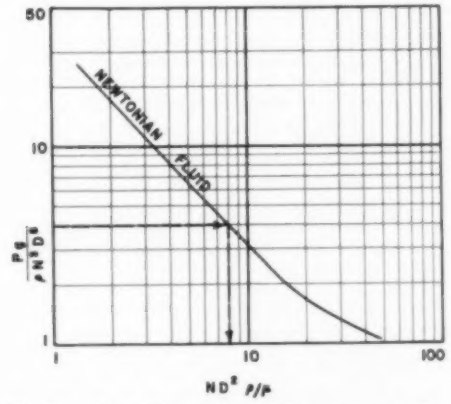

Fig. 14.—Typical Reynolds number power number curve for a Newtonian fluid.

mean that they will be very slow settling out of suspension.

Settling velocity, of course, is measured in the fluid under consideration. If the fluid has non-Newtonian characteristics, then the determination of the proper settling velocity to be used must be carefully considered. These cases usually involve laboratory examination.

PROCESS REQUIREMENTS

In giving process requirements for suspension operations, it is desirable to distinguish between the characteristics of the solids, whether they have a finite settling velocity or not. It is also desirable to distinguish between a suspension of solids and a requirement for breaking up agglomerates of solids. If the solids are difficult to wet out, this should also be noted.

In the discussion below, three separate categories are treated: (*a*) suspension of particles of essentially zero settling velocity, (*b*) suspension of solids with a finite settling velocity, (*c*) dispersion of solids and agglomerates.

Essentially Zero Settling Velocity.—In these processes, the major requirement is to produce a uniform dispersion throughout the entire medium. For batch operation, it is usually a matter of determining the time required to obtain a uniform suspension. If the solids are difficult to pull in from the surface, an impeller is often placed near the surface to aid in this operation. However, this also induces air into the system. Once uniformity is achieved, there is no further advantage in keeping the mixer on.

Propeller-type mixers come in sizes up to 3 horsepower and can handle viscosities up to 5,000 cps. in tanks up to 500 gallons, and tanks up to 5,000 gallons in volume with water-like fluids. Normally, gear drive mixers at speeds from 280 to 420 r. p. m. are used in these operations. Tank proportions of liquid depth equal to tank diameter are used, but other proportions may be used if required.

If the power requirement is above 3 horsepower, turbine units are normally used. These are equipped with either fan turbines or flat blade turbines.

Finite Settling Velocities.—In this case, solids are picked up in suspension rapidly but may settle out if the mixer is turned off. The degree of uniformity produced is a function of the power applied to the system, but if the settling velocities of any of the

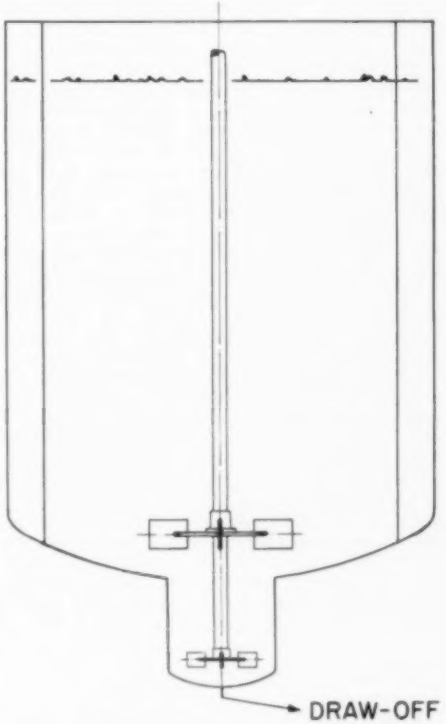

Fig. 15.—Schematic drawing of tank construction for draw-off.

particles are above 1 foot per minute, there may be gradients in concentration throughout the tank unless relatively high power levels are used. If the settling velocities are below 1 foot per minute, the solids will be essentially uniform throughout the entire tank.

As in the case above, gear drive propeller units are commonly used in applications requiring 3 horsepower or less, while turbine units are used for the more difficult applications.

One problem which often occurs is to produce a uniform suspension in the tank and maintain this suspension while material is drawn off to a filling or packaging machine. Sometimes this can be done by putting dual impellers in the system and placing the lower impeller very near the bottom of the tank.

However, the very effective way is to place a small tank at the bottom of the main tank shown in Fig. 15. An impeller of sufficient size is placed in the small tank to maintain a uniform suspension while the draw-off proceeds. The mixer is operated through draw-off, keeping the solids suspended throughout the entire process.

Solids Dispersion.—In case there are agglomerates to be broken up, it is desirable to use higher shear rates. In these cases, direct drive 1,750 or 1,150 r. p. m. propeller-type units are used. If additional dispersion is required, units operating in the 3,000 to 4,000 r. p. m. range may be employed with an impeller designed for high speed operation. These high speed shear rates will break down agglomerates in the system. It is essential in describing the mixing operation to state whether agglomerates are to be dispersed or whether it is primarily a matter of suspending agglomerate particles.

Air Entrainment.—There are times when the entrainment of air in the batch is of particular concern. In case it is desired to keep the degree of air entrainment to a minimum, at least 1 impeller diameter of coverage should be provided on the upper impeller. If the tank is equipped with wall baffles, baffles which extend across the surface also are often helpful. Figure 16 illustrates the arrangement of these baffles.

In case there is a variable liquid level, it is sometimes desirable to use multispeed units, or to arrange for additional baffles at the lower liquid level.

Variable speed is discussed further under "Equipment."

Crystal Characteristics.—If the particles are sensitive to fluid shear, then the fluid shear component in the system must be carefully controlled. It is usually desirable to investigate the effect of fluid shear on crystal growth on small laboratory equipment. High speed direct drive mixers should be avoided if fluid shear is detrimental. The use of the slow speed gear drive propeller mixers and the still slower speed turbine type mixers is required if fluid shear rates must be kept to a minimum.

In crystallization processes, the overall circulating capacity must be carefully related to fluid shear to provide the proper particle size.

PILOT PLANTING

Purpose of Pilot Planting.—The ultimate aim of all process designers is to design a process which successfully meets the requirements of the plant.

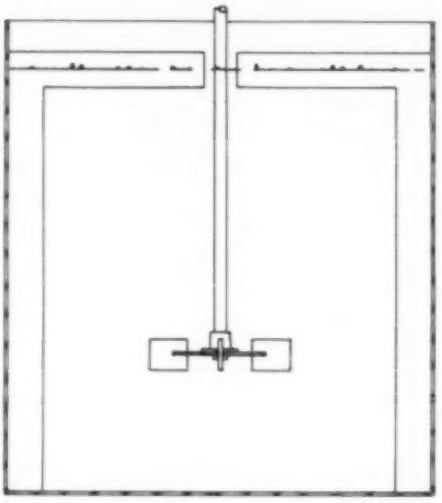

Fig. 16.—Arrangement of baffles across the surface to prevent air entrainment.

To meet this aim, a complete investigation of the current mixing knowledge relative to the application should be made. By the most practical method, data should be extrapolated to the plant size process. At this point, the accuracy of this extrapolation should be determined and the possible ranges of mixer sizes estimated.

Then, the possible economic advantages to be gained by a thorough knowledge of mixing variables in the process should be estimated. This will give an adequate basis for deciding on the merits and extent of a proposed pilot plant study.

Pilot plant studies can range from one "spot check" in a six-inch tank, to an extensive project covering several months of work with equipment in several tank sizes.

A Summary of Pilot Plant Procedures.—Regardless of the complexity of the process, a basic pattern of investigation must first be followed. Holding all other variables constant except impeller speed and diameter, a basic series of runs must be made. These experiments measure the effect of (*a*) horsepower (three to six runs), and (*b*) impeller diameter to tank diameter ratio (one to three runs). The interpretation of the results from these basic runs is discussed below. In making these runs, there are several rules that should be followed:

1. The impeller must be geometrically similar to the proposed full scale installation.

2. The tank proportions must be geometrically similar. The only exception to this rule is that the ratio of liquid depth to tank depth need not be held constant. Corrections for this factor can usually be made to the basic pilot plant runs.

3. Horsepower must be varied in at least four steps over a ten-fold range as a minimum. The total speed must thus be approximately twofold. Wider variations in power are desirable.

4. When varying other variables, such as gas flow, liquid flow, etc., at least threefold variation between steps must be made, so that pronounced differences in process result will occur.

Effect of Horsepower.—The basic runs to be made first are to relate process result to mixer horsepower. Pilot plant impellers and tanks should be geometrically similar to projected full scale equipment if useful results are to be received. Pilot plant impellers whose power characteristics are known, enable horsepower to be calculated from speed measurements, and elaborate dynamometers are usually not necessary.

These studies should be carried out from zero horsepower up to a value sufficient to cause a leveling out of the process result. From the slope of this curve of process result *versus* mixer horsepower, many preliminary observations can be made which will point the way to proper follow-up experiments, see Fig. 17.

Effect of D/T Ratio.—The ratio of impeller size to tank size is related to the ratio of fluid flow to impeller velocity head. In the remarks above on the effects of horsepower, these runs many times can be neglected or calculated from other known factors. If necessary, the effect of D/T should be evaluated at either constant horsepower and the result measured, or at constant result and the horsepower measured, to achieve this result.

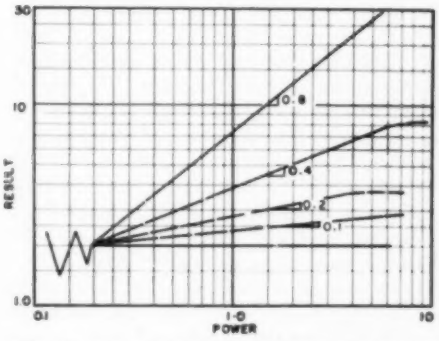

Fig. 17.—Process result *versus* horsepower for pilot plant studies. (Interpretation of pilot plant results, effect of power; all conditions constant except power.)

Measurement of Power Input.—The power input to a mixing vessel can be measured by several different methods. Each of these methods has its own advantages and disadvantages and these are discussed below:

Calibrated Impellers.—In an operation where the fluid properties can be accurately evaluated, power consumption can be obtained from power curves and calibrated pilot plant turbines. Therefore, all that is necessary is to measure impeller speed and position and the factor to apply for fluid properties. This is by far the most common method, the most convenient, and the least costly. A flat blade turbine (Fig. 4) can be supplied with calibration curves.

Dynamometers.—The simplest dynamometer consists of a motor mounted on a trunion bearing with an attached pulley running to a scale. The torque reaction on the impeller gives an equal and opposite reaction on the frame of the motor.

Electric Power Input.—For small scale equipment, it is often very difficult to get accurate power measurements from watt-meter readings. The problems normally encountered are: operation below one-third of the rated motor capacity, excessive no-load friction losses, and unknown losses through variable speed devices. If watt-meter readings of high accuracy can be taken and if the losses of motor and drive assembly can be obtained with reasonable accuracy, this should offer an acceptable calculation for power input.

SCALE-UP

Similarity relationships are of great value in scale-up calculations. Since the fluid regime controls the mixing result, we can use the laws of hydraulic similarity. Two types of similarity are important, geometric similarity and dynamic similarity. Geometric similarity is the first requirement in all scale-up procedures. Dynamic similarity deals with fluid forces. There are four of these fluid forces.

The first of these forces is inertia force, F_I. This is the force we apply to the system. It is related to the power, speed, and diameter of the impeller.

The other three forces are the ones which resist the accomplishment of our process, namely viscosity, F_v, surface tension, F_σ and gravity F_g. Gravitational forces are usually negligible if baffles are used in the tank. Surface tension affects the formation of surface area in a two-phase system, but is usually not of great importance in those systems. Viscosity is the predominant force which influences the fluid regime in the vessel.

The ratio of inertia force to the viscous force, F_I/F_v, is the Reynolds number and has the value $ND^2\rho/\mu$. This is the ratio of what we apply to the system divided by the resisting force of viscosity. This determines the motion of the fluid in the tank.

The process result is represented by R. The process result alone cannot be correlated with Reynolds numbers, since R has units and depends upon the process definition given to it, while the Reynolds number is a dimensionless ratio. When a dimensionless ratio involving $[R]$ cannot be used, a second method of analysis for a particular process in both a small and a large scale in which the fluid properties are constant in both scales, takes the form of

$$R = f[F_I][\text{scale factor}] \qquad \text{(Eq. 6)}$$

Geometric similarity is implied in this relationship. The group representing the inertia force can either be speed, peripheral speed, power, or power per unit volume, or even a dimensionless number such as Reynolds number.

The choice of which index to use is determined by the scale factor that results. The choice of horsepower per unit volume for F_I has several advantages. One advantage of this choice is that the scale factor varies the least above and below unit of any group that can be chosen.

For scale-up, the factor must be known either by previous information, such as research or plant scale data, or must be determined as part of the pilot plant procedure.

EQUIPMENT

There are three classes of equipment normally used in pharmaceutical solid suspensions. These are classified by the method of mounting the mixer

and the impellers used: (*a*) portable propeller type mixers which mount to the side of the tank; (*b*) fixed mounted propeller type mixers, mounted to fixed mounted beams in the tank; and (*c*) top-entering turbine type drives, fixed-mounted and using turbine type impellers.

Portable Mixers.—Portable mixers are used in sizes up to 3 horsepower. They may either be direct drive, 1,150, or 1,750 r. p. m., or gear drive, 350 r. p. m.

Gear drive units give a high pumping capacity and relatively low level of fluid shear. They are normally used where suspension operations are to be carried out. Either single or dual impellers may be used depending upon batch proportions.

The direct drive units have higher shear rates and are used where dispersion operations are important. These units are normally equipped with dual impellers.

Portable type units are usually mounted off-center so that baffles are not required. They are used in tanks up to 3,000 gallons for low viscosity suspension operations and are used in small volumes in viscosities up to about 5,000 cps.

Fixed Mounted Propeller-Type Mixers.—These units are similar to portable mixers except that they are mounted directly to the tank head or mounting beams. They are used for either open or closed tank operations. Their range of process applications is similar to the unit discussed above. Normally they are mounted in the angular off-center position, although they may also be mounted on center with baffles.

Fixed-Mounted Top-Entering Mixers.—Turbine-type units are available in any size from 1 to 500 horsepower. They are used for larger tanks, higher viscosities, and difficult suspension operations.

From 1 to 3 horsepower, turbine-type units are used on difficult operations requiring their rugged construction.

Above 3 horsepower, they are normally required in all mixing applications, and may be equipped with change gears which allow the selection of various speeds in case the application should change.

Depending upon the liquid level to the tank diameter ratio, single or dual impellers are used. Either fan turbines or flat blade turbines, shown in Figs. 2 and 3 are used in these applications. The unit is normally mounted on center with baffles at the wall. As mentioned previously, the baffles are usually set off the tank wall about two inches to prevent solids building up at that point.

Variable Speed.—In case variable speed is desired, there are many different varieties available. Basic classifications of variable speed devices follow:

I. Fluid Drives
   - A. Air
   - B. Hydraulic transmission
   - C. Fluid couplings

II. Mechanical Drives
   - A. Separate belt drive
   - B. Integral belt drive
   - C. Metallic traction

III. Electrical Drives
   - A. Multispeed motor
   - B. A. C.-D. C. drives
   - C. Magnetic coupling
   - D. A. C. drives
     - Variable voltage
     - Shifting brush
     - Variable frequency

For use on small equipment, up to about 3 horsepower, air motors, multispeed drives, and alternating current variable speed units are common.

Multispeed motors give effective reduction in power when splashing is to be avoided. Most of the other types of drives are in-motion control and are used for more specialized conditions.

GLOSSARY OF TERMS

D, Impeller diameter; du/dr, shear rate; F_i, inertia force; F_g, gravity force; F_v, viscosity force; $F\sigma$, surface tension force; f, function of; g, gravitational acceleration; H, impeller head; hp., horsepower; K, K', constant in viscosity, shown in Figs. 12 and 13; N, impeller speed, r. p. m.; N_P, power number, ratio of inertia force to mass $\times$ acceleration $P_g/(\rho N^3 D^5)$, dimensionless; N_{Re}, Reynolds number, ratio of inertia force to viscosity force, $ND^2\rho/\mu$, dimensionless; P, power; Q, impeller flow, volumetric fluid displacement of impeller; R, process result; T, tank diameter; μ, viscosity; σ, surface tension; τ, shear stress.

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Performance and Problems of Pharmaceutical Suspensions

By KENNETH J. FREDERICK

The increasing use of physical chemistry data in the evaluation of the stability and therapeutic efficacy of medicinal suspensions is discussed and illustrated. The factors involved in the selection of the dispersed phase and the changes in its characteristics which can occur during storage are emphasized.

THIS PAPER will consider how suspensions can best be evaluated to see how well they meet the purpose for which they were designed. The performance check of suspensions will not be reviewed from the standpoint of the control chemist who is following a standard set of procedures on a series of production samples and whose curiosity and need are generally satisfied when he has a yes or no answer. Rather, suspensions will be examined at the research and development stage in their evolution and through the eyes of the pharmacists who want to be certain that they have the very best possible formulation for the job at hand.

PRESENT EVALUATION PLAN

The major aspects of present practice in performance checks on suspensions can be outlined as follows:

1. Shelf stability
   - *a.* Gross physical factors
   - *b.* Drug potency
2. Pharmacological evaluation
   - *a.* Toxicity
   - *b.* Therapeutic value—blood level etc.
3. Patient acceptance
   - *a.* Oral suspensions
     - Taste panel
   - *b.* I. M. suspensions
     - Pain upon injection
     - Pain persisting after injection
4. Clinical reports

In the case of a new formulation, if the research pharmacist considers the chemical and physical stability of the suspension to be satisfactory, if the pharmacologist finds the preparation is relatively nontoxic and has sufficient activity in animals to justify clinical trial, if the clinician asked to evaluate the test formulation finds it to be therapeutically effective, and if the patient and/or nurse accept the product without complaint, the project is considered a success and the curtain goes up.

INCREASING USE OF PHYSICAL DATA

In recent years physical chemistry data have been playing an increasingly important role in this picture of suspension evaluation, particularly in those areas which are within the province of the research pharmacist. These areas include prediction of formulation and drug stability, and correlation of physical characterization of a formulation with its therapeutic effectiveness. There are a number of examples of this use of physical data in the specific field of oral and intramuscular preparations which are worth mention.

First, though, a comment is in order on the importance to the research pharmacist of securing as much basic data on a new drug as possible at the start of the formulation program. In some companies a pretty fair job is now being done along this line; in others, the pharmacists start working with a new drug with very little data on (*a*) its purity, (*b*) its approximate solubility in water and other solvents approved for clinical use, (*c*) the influence of pH upon solubility, and (*d*) the general stability pattern of the drug in the temperature range, 5–75°. Pharmacists need this sort of information on a new drug along with knowledge of any special precautions which need to be exercised in its handling before beginning formulation work.

ORAL SUSPENSIONS

Now, in regard to oral suspensions, the physical tests worth doing are the following:

1. More complete examination of properties of solution phase with particular emphasis on drug stability and taste alteration.
2. Microscopic and X-ray study of solid phase.
3. Check on suspension viscosity changes.
4. Gas chromatographic studies in odor control.

It is common practice in judging both physical stability of a formulation, and in testing retention of drug potency, to examine only the intact suspension. A more meaningful evaluation of both factors would result if, in addition, separate studies of both the clear solution and suspended phase were undertaken.

Build-up of degradation products in the solution of the suspended drug, for example, can oftentimes be spotted readily by spectrophotometric changes, pointing positively to incipient loss of drug potency. This point frequently is hard to determine when the total drug activity is measured and a decision must be made whether the reported drop from 102 to 99.5% of theory is real.

Further, the beginning of a real and serious alteration of taste may be detected on the separated solution phase which may be hard to pick up on the intact suspension. The taste buds belonging to the taste panel will respond more consistently in sampling serial samples of dilute solutions of the drug

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in the absence of particulate matter, and the physical chemist operating on the solution alone can answer unequivocally whether hydrolysis of the organic chemist's pet ester has commenced.

A careful study of the solid phases from stability samples will improve greatly the correctness of physical stability prediction. Slowly developing changes in particle size distribution, crystal habit, or crystal system, will shine forth as red lights and can be discerned many times before gross changes in viscosity become evident, although don't forego viscosity check for this or for any reason.

As the third item, there is the use of gas chromatography in the study of odor control. The author has had no first-hand experience in this area in the laboratory, as far as pharmaceutical suspensions are concerned, but he recalls vividly the number of times in years past when his young sons have choked on foul-smelling penicillin suspensions which his pharmacist friends insisted were 100% okay. In the combined area of odor and taste control, the gas chromatograph is certainly a powerful tool which should not be overlooked.

INTRAMUSCULAR SUSPENSIONS

The story on intramuscular suspensions is much the same. Here, the physical testing can include:

1. More complete examination of properties of solution phase with particular emphasis on drug stability and tonicity changes.
2. Microscopic and X-ray study of solid phase.
3. Check on suspension viscosity changes.
4. Rate of disappearance studies from site of injection and use of radioisotopes.
5. Animal and laboratory studies of depot-forming tendencies.

The separate testing of solution and solid phases is very much in order and for the same reasons just discussed. In examining the solution phase another property comes into the picture, however, when intramuscular preparations are involved, *viz.* tonicity. The solution has probably been designed by the pharmacist to be isotonic or reasonably close thereto. Serious departures therefrom need to be guarded against to insure a minimum of muscle tissue irritation effects. This also is another way of checking on drug degradative effects.

There are many ways in which radioisotopic tracers can be used to check on the performance of intramuscular suspensions. Disappearance studies from the site of injection in test animals is an ideal example, especially for a repository-type preparation. Such studies can be extremely useful to the research pharmacist who can explore rapidly the full gamut of variables at his command.

INJECTION DEPOTS

Where a radioisotopic tracer of sufficient penetrating power is not available, consideration can be given to other procedures for evaluating depot-forming tendencies. A few comments on a published report from the author's laboratory dealing with an intramuscular procaine penicillin G product (1) will serve best to illustrate this point.

In this case it was known at the outset from the years of clinical use which preceded the detailed physical chemical study that the product was a very effective repository preparation which gave excellent blood levels for more than seventy-two hours from a single injection of 600,000 units/ml. It was suspected that a hard core of procaine penicillin G crystals developed in the muscle near the site of injection as a result of a diffusion away from the site of the excess water in the product.

After a few preliminary injections into inexpensive pork and beef roasts showed that depot formation was truly involved, pharmacologist friends were asked to help. Some of the compact cores which they extracted from test rabbits were shown in our earlier report (1). Since it was not feasible to continue this rabbit muscle work for all of the dozens of test suspensions on hand, a search for an *in vitro* means of measuring depot formation was made. This search led to the use of 2% gelatin gels as a suitable injection medium in place of rabbit behinds. For the particular product with which we were dealing 2% gels were quite equivalent in action to rabbit muscle. This equivalency, however, most certainly will not hold for products where the volume of injected suspension and the ratio of total solids to total and effective free water are materially different.

The *in vitro* test which was developed from the depot studies was of great help to us in the comparative evaluation of both research and competitive samples.

Such a fundamental approach may well pay off in other problems of pharmaceutical formulation and lead to *in vitro* screening tools of inestimable value to the research pharmacist charged with producing unbeatable formulations having patentable features. This is all part of the new performance check of suspensions which the study of physical pharmacy is bringing about.

SELECTION OF POWDER

There are other problems met in suspension formulation work, aside from the basic questions of settling and caking. Attention needs to be centered on the dispersed phase, the suspended drug, and on (*a*) those factors which are important in selecting the particular powder which is used, and (*b*) the changes in characteristics of the solid phase which time often brings about.

PSD.—The parameter over which the pharmacist has the most control in selecting the sample of drug to be suspended is the degree of fineness or coarseness of the material. He may be dealing with a crystalline product carefully grown to a narrow size range, but more likely he is dealing with a ground material resulting from a milling or micronizing operation. The material probably has been classified to remove the oversize particulates, and perhaps, but less likely, the excessive fines. In any event, although all pharmaceutical powders may be characterized by a figure for the average particle size, there will be present actually a distribution of particle sizes. This distribution can be measured in various ways, but only with great difficulty. The PSD or particle size distribution is expressed either in number per cent values or in weight per cent figures. The latter is usually used, and is the more meaningful.

Surface Area.—Instead of PSD measurements many are using successfully for control purposes

surface area data expressed as the total particulate area per unit weight which is available for contact with the vehicle. The unit most frequently used is sq. cm./Gm. and values run from 2,500 sq. cm./Gm. for a coarsely milled material to 75,000 sq. cm./Gm. for an extremely fine micronized powder.

Surface area measurements, aside from the ease with which the measurements can be made, and their reproducibility, in contrast with PSD data, are much more useful in suspension formulation work, especially when the solids/water ratio is high and the resulting suspensions are thick, lack the ability to flow, are non-Newtonian, and show thixotropic behavior. For in such instances an abundance of extremely fine particles which appears so insignificant in any weight per cent PSD, and yet which plays such an important role in determining the characteristics of a viscous suspension, is readily detected by a high surface area reading.

However, surface area data are not the answer to every prayer, and so some information on PSD is necessary if a thorough study is to be made correlating powder characteristics with suspension properties and clinical effectiveness. Recent literature on this subject shows clearly the importance of powder characteristics in suspension formulation work, and spells out the rewards which stem from such basic considerations. Two examples will serve to illustrate this point.

Influence on Blood Level.—First, another reference will be made to our study of high-solids procaine penicillin G suspensions. In this work a relationship was found between gel depots and clinical blood level data. In addition, the viscometric or rheological behavior of our best suspensions was found to involve particle size distribution, surface area, and solids/water ratio data. In short, we were able to correlate blood level data with the physical properties of our suspensions and to correlate the latter in turn with the characteristics of the starting penicillin powders. A sounder approach to our formulation studies in this field, improved patent protection, and establishment of simple routine control tests to insure uniformity of product, all resulted from this excursion into basic research.

To complete this reference on a more specific note, as far as powder characteristics are concerned, our study revealed that: (*a*) surface area measurements on the powder by means of the Blaine fineness tester was a feasible and adequate control on our micronizing operation, and (*b*) our most manageable and therapeutically effective products resulted when we had a relatively broad distribution of crystal sizes readily obtained by a blend of milled and micronized materials.

A similar study has been reported by Buckwalter and Dickison (2) on the effect of vehicle and particle size on the absorption of intramuscular procaine penicillin G suspensions. Their study brings out very sharply two facts: (*a*) large particles extend the effectiveness of dilute intramuscular suspensions by virtue of slower dissolving action, and (*b*) small particles appear better when aluminum monostearate is present.

The latter observation probably stems from the incipient depot-formation brought about by the monostearate and is the result of the marked cementing action possessed by the fine particles. Whatever the explanation, here in a 300,000 units/ml. preparation, as in our study involving 600,000 units/ml. products, a combination of crystal sizes offers distinct advantages.

Consideration has been given thus far to the importance in suspension formulation work of selecting carefully the characteristics of the dispersed phase. The variables have been pretty much under our control and in each instance there has been a strong element of predictability.

CRYSTAL CHANGES

A much more difficult area, where black magic so often appears to enter the scene, occurs when changes in crystal size and shape take place either soon after the suspension is put together or slowly and unsuspectingly. Obviously, it will do little good to tailor a dispersed phase to exact specifications, if elments of growth and transformation subsequently upset everything.

This question of physical stability of the dispersed phase in suspension is an extremely important problem. In this paper only a brief outline of what is involved can be drawn. Consideration will be given first to crystal growth exclusive of polymorphic and similar changes, and then later to the fundamental problems of polymorphism and solvation.

Crystal growth in suspensions, leaving out polymorphic and solvation transitions, is generally not a serious problem. If in our formulation of suspensions, in the laboratory or in the plant, the time of mixing were long enough and the temperature were constant enough to reach true equilibrium—as in a physical chemical determination of solubility—and if the temperature of filling and storage were constant and identical to the temperature of mixing, and if there were no elements of chemical instability involved and no effect of particle size upon solubility, there should be no alteration in the characteristics of the dispersed phase with time.

In actual practice there are many possible crystal changes which can take place in suspensions. Excluding for the moment polymorphic and solvation transformations, these changes may embrace the following:

A. No change in crystal habit.
   1. Slow approach to equilibrium.
      Some loss in fines.
   2. Decomposition of drug possibly leading to salting-in, salting-out, and/or pH effects.
      Minor changes in PSD.
   3. Cycling of shelf temperature.
      Could seriously alter PSD.
      Depends on many factors.

B. New Crystal habit.

Size Effects.—The first effect, involving a slow approach to equilibrium, generally doesn't cause much trouble and so can be neglected. And the alteration of the characteristics of the dispersed phase brought about as a consequence of the chemical instability of the drug also probably isn't too troublesome, since the magnitude of the salting-in or salting-out effects resulting from accumulation of degradation products isn't going to be sufficient to alter appreciably the particle size distribution, unless a pH change occurs simultaneously and this, in itself, has a marked effect on solubility. However, the salting-in effect may exert an autocatalytic

effect on the rate of decomposition of the drug, but this is a different story.

The third item on the other hand, alternation of shelf temperatures, can at times be very significant. Its full import, of course, depends on (*a*) the details of the temperature cycle, how much of an increase or decrease is there, how often does this take place, and how fast; (*b*) the slope of the solubility-temperature curve for the dispersed phase, the greater the slope, the more likelihood there is of change; and (*c*) the rates of dissolution and crystallization. And here, of course, the suspending agents used to increase viscosity and reduce settling, the coating efforts which may have been made to slow down chemical degradation, and so on, all come into the picture and collectively serve to minimize the effect of shelf temperature cycling. In the prediction of shelf stability, chemical and physical, more cycling of storage temperature should be used. In the case of suspensions, this practice would help a great deal to uncover marked crystal growth changes capable of causing trouble in the field.

Up to this point, possible alterations in particle size distribution which involve no change in crystal habit have been discussed. The magnitude of these alterations quite obviously depends on the solids/water ratio of the suspension under study, the more solids present, the less significant on a percentage basis will these effects be.

Habit Change.—If on this picture is superimposed the element of crystal habit, a supplementary driving force which can be mighty important is brought into play. The term crystal habit refers to the external shape of the crystal and is governed by the relative sizes of its characteristic faces. For a particular crystalline material the interfacial angles and melting points of the various habits are the same. The habit taken by a crystal, be it needles, plates, cubes, rods, prisms, etc., is governed by factors which dictate the rate of growth of the crystal. Rate of cooling, extent of agitation, size and number of nuclei, and all elements which affect the degree of supersaturation of the solution bathing the surfaces of the crystal have a profound effect on crystal habit formation. Also playing a role are such things as surface tension, container material, pH, solvent, and particularly important, impurities. Crystals used to prepare pharmaceutical suspensions have generally been prepared under environmental conditions far different from the vehicles and containers used in the formulation work. If a different crystal habit is favored by the formulation environment than by the production situation, there is a conversion tendency which may prove to be quite a thorn in suspension work. How serious this problem might be is impossible to say, but we should be on the alert for it and remember two facts: (*a*) habit preferences will generally come into the picture only if one of the factors cited earlier is present; and (*b*) where a change in habit is involved, this factor may be expected to increase the seriousness of the changes in particle size distribution which will occur.

Solvation.—Problems also arise where distinct new crystalline entities are formed in suspensions during storage as a result of solvation and polymorphic transformation. The principles involved in solvation or desolvation are pretty well understood, although often neglected in the heat of battle. If anhydrous crystals of a drug are placed in an aqueous medium where the solid phase in equilibrium with the solution is a hydrate, there definitely will be a conversion. Similarly, if a hydrate is used under conditions where the anhydrous drug is the solid phase in equilibrium with the solution, a transition will occur. The story is obviously the same for solvates other than hydrates. In all cases the rate of conversion is unpredictable and may be very slow, or very fast.

Changes of this sort frequently occur throughout formulation and stability testing programs. Basic information on new drugs, therefore, should include data on all solvates of interest, and particularly, hydrates.

Polymorphism.—Finally, consideration will be given to the subject of polymorphism. Polymorphs are simply different crystal structures, i.e., different space-lattice arrangements, of the same compound, usually indicated by different melting points. The temperature, solvent chosen for crystallization, and rate of cooling are important factors in determining which polymorph is obtained. Since polymorphs of a drug dissolve to give identical solutions, at this stage they are indistinguishable and lose all significance. However, in the crystalline state polymorphs are distinct entities, clearly distinguishable by X-ray diffraction fingerprints and other crystallographic data and each possessing its own list of physical properties. The fact that polymorphs of a drug differ in their solubility properties is one of the most important elements in this whole picture. It has been said by McCrone (3) that all organic substances exist in a number of polymorphic states and that the number of polymorphs reported in the literature for any organic chemical simply reflects the degree of effort which has been applied in the basic study of this chemical. If all of the polymorphs described in the scientific and patent literature for established drugs were listed, including all of the polymorphs known to exist for present commercial antibiotics, the truth of McCrone's contention would be established.

It might be interesting to note in passing the number of known polymorphs of a few established drugs: barbital, 4; cortisone acetate, 3; nicotinamide, 4; progesterone, 5; riboflavin, 3; and sulfathiazole, 3. This list is merely illustrative and is by no means complete. The figures are probably all on the low side.

There are many ways in which polymorphs can be detected. McCrone (3) is a good reference to use for this purpose. But is it necessary, is it feasible, to study exhaustedly each new drug to determine how many polymorphs may exist? The author's answer to this question is a faint-hearted no. A systematic survey of polymorphs for each new drug would be prohibitively expensive. For this reason it is more important to proceed with the suspension formulation work and then to check thoroughly during the stability test program by microscopic and X-ray study for changes in the dispersed phase. Such a procedure should certainly uncover those polymorphs which are born trouble-makers in suspension work.

Occasionally a polymorph will be found whose properties are such that it stands out in clinical effectiveness above its brethren. When this occurs, it is due undoubtedly to the differences which can and do exist among polymorphs in water solubility,

water-oil partition coefficients, and in rate of solution, and to the dominant role these properties can exert in some instances in dictating which polymorph shows good physiological activity and which receives an inactive label. The commercial factors in such a picture are many and obvious. While a basic study of polymorphism on a particular new drug may pay rich dividends, don't expect a jackpot with every pull of the lever.

There's one other aspect of polymorphism which deserves brief mention. Polymorphs are said to be enantiotropic, when each has a definite range of stability, and the change from one form to another takes place at a definite temperature in either direction. The two solids coexist in equilibrium at the transition temperature, a temperature which is below the melting point. On the other hand, polymorphs are said to be monotropic when one of the polymorphs is stable and the other or others are metastable over the whole range of their existence. The polymorphic change can then take place in one direction only, i. e., from metastable to stable, the stable form always having the lowest free energy and the highest melting point. Most examples of polymorphism among drugs are of this latter type.

The rate of conversion of a particular metastable polymorph to the stable form may be so fast as to cause trouble in formulation work, or it may be so slow as to make this metastable variety "stable" for all practical purposes. When the rate is fast, or relatively fast, trouble will usually arise if a metastable form is used in ignorance. On the other hand, where the rate of conversion is very slow, metastable forms are feasible and frequently more to be desired than the stable forms because of their generally higher solubilities. A ecent study has shown that several commercial preparations of sex hormones contain the drugs as metastable polymorphs. However, a metastable polymorph which converts so slowly to the stable form as to give the impression of stability and to justify commercial exploitation may ultimately be benched when a friendly competitor uncovers the true stable polymorph and finds its lower solubility of advantage in reducing the extent of degradation in suspension.

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Notes

Determination of Quaternary Ammonium Compounds

By JOHN J. HEFFERREN and CAROL DIETZ

The official potassium ferricyanide assays of quaternary ammonium preparations can be modified to include a potentiometric titration with silver nitrate instead of the suggested iodometric method for the determination of the excess ferricyanide. Sodium nitrite interferes with the ferricyanide assay methods.

THE REACTION of potassium ferricyanide and quaternary ammonium compounds to form a water-insoluble precipitate has been used in official assays for benzalkonium chloride (1) and benzethonium chloride (2) and can be used for other quaternary ammonium compounds. In these assays an excess of potassium ferricyanide is added to a solution of quaternary ammonium compound, zinc sulfate, and acetate buffer. The quaternary ammonium-ferricyanide precipitates immediately, and the excess ferricyanide in a filtered aliquot is then determined by the standard iodometric procedure. The reactions involved may be represented as follows (3):

$$3\ R_4NCl + K_3Fe(CN)_6 \rightarrow (R_4N)_3Fe(CN)_6\downarrow + 3\ KCl$$

$$2\ K_3Fe(CN)_6 + 3\ ZnSO_4 + 2\ KI \xrightarrow{H^+} Zn_3K_2[Fe(CN)_6]_2\downarrow + I_2 + 3\ K_2SO_4$$

$$I_2 + 2\ Na_2S_2O_3 \xrightarrow{H^+} Na_2S_4O_6 + 2\ NaI$$

The iodometric procedure is successful because of formation of the water-insoluble zinc salt which shifts the ferri-ferrocyanide equilibrium to the right (4).

The potentiometric titration of the potassium ferricyanide with silver nitrate (5) can be readily applied to the determination of the excess ferricyanide in these quaternary assay procedures (Fig. 1). The potentiometric titration avoids any color interferences; however, a preliminary titration with

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Fig. 1.—Potentiometric titration curves of benzethonium chloride (——) and potassium ferricyanide (- - -) in acetate buffer with silver nitrate.

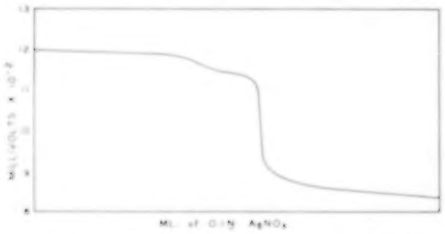

Fig. 2.—Potentiometric titration curve of potassium ferrocyanide in acetate buffer with silver nitrate.

silver nitrate of the halides in the quaternary ammonium preparation must be run since these silver nitrate titratable compounds will also be present in the filtered aliquot containing the excess ferricyanide. The halide determination serves as a blank for the titration of the excess ferricyanide and provides information on the halide content of the quaternary. Such information is useful in that some quaternary ammonium agents contain significant quantities of inorganic halides.

Many commercial quaternary ammonium solutions, which are intended for the chemical disinfection of dental and other instruments (6), contain sodium nitrite as a rust inhibitor. The presence of an antioxidant, such as nitrite, causes a number of competitive reactions to occur in the iodometric determination of potassium ferricyanide. Sant (7) used the reaction between nitrite and ferricyanide as a method for the determination of nitrite.

Nitrite also interferes with the silver nitrate determination of ferricyanide. The titration with silver nitrate of potassium ferricyanide in the presence of sodium nitrite and acetate buffer

$$2\ Fe(CN)_6^{3-} + NO_2^- \rightarrow 2\ Fe(CN)_6^{4-} + NO_3^-$$

$$Fe(CN)_6^{4-} + 4\ AgNO_3 \rightarrow Ag_4Fe(CN)_6\downarrow + 4\ NO_3^-$$

indicates that the ferricyanide is converted almost quantitatively to the ferrocyanide, thereby consuming four equivalents of silver nitrate. Titration of potassium ferrocyanide in acetate buffer similarly consumes four moles of silver nitrate (Fig. 2). Sodium nitrite has no effect on the titration of ferrocyanide or the halide titration with silver nitrate.

Quaternary ammonium compounds, including benzethonium and benzalkonium chloride, react with potassium ferrocyanide in acetate buffer to give a white to cream colored precipitate. The characteristics of this precipitate are very similar to the comparable, yellow, quaternary ammonium-ferricyanide precipitates. The precipitates appear to consist of four molecules of the quaternary ammonium compound and one of the ferrocyanide moiety. Preliminary attempts to quantitate this ferrocyanide reaction for reaction conditions similar to those suggested for the ferricyanide reaction

$$4\ R_4NCl + K_4Fe(CN)_6 \rightarrow (R_4N)_4Fe(CN)_6\downarrow + 4\ KCl$$

have resulted in somewhat inconsistent and high assay values. This was presumably due to the character of the ferrocyanide precipitate and the equilibrium of the reaction.

EXPERIMENTAL

Apparatus.—A Beckman Zeromatic pH meter equipped with a silver billet combination electrode, No. 39187.

Reagents.—All reagents were of analytical grade. Potassium ferricyanide and ferrocyanide were each standardized against 0.1 *N* silver nitrate and were stored in a brown bottle. These solutions should be restandardized before using. The acetate buffer contained 26 Gm. of sodium acetate and 22 ml. of acetic acid in sufficient water to make 100 ml. of solution.

Procedure.—*Halide Determination.*—Dissolve about 1.5 meq. of quaternary ammonium compound in about 50 ml. of water, or take an equivalent amount of commercially prepared solution and dilute with water. Add 5 ml. of acetate buffer and titrate potentiometrically with 0.1 *N* silver nitrate. The number of ml. times the normality of silver nitrate is equal to the number of meq. of halide present in the quaternary ammonium compound.

Quaternary Ammonium Determination.—Transfer to a 100-ml. volumetric flask an amount of quaternary ammonium compound equivalent to about 0.5 meq. Add 5 ml. of acetate buffer and exactly 50 ml. of 0.1 *N* potassium ferricyanide. Dilute to the mark with water and mechanically shake at room temperature for one hour. Filter the mixture through a hard filter paper, discarding the first 20 ml. of filtrate. Titrate potentiometrically a 50-ml. aliquot of the filtrate with 0.1 *N* silver nitrate. The ml. times normality of silver nitrate minus the meq. of halide equivalent to the amount of quaternary ammonium compound used are equal to the meq. of potassium ferricyanide in excess. The meq. added, minus the excess of potassium ferricyanide is equal to the meq. of quaternary ammonium compound.

Triplicate assay results with benzethonium chloride monohydrate (mol. wt. 466.11) were: 96.5, 93.8, and 97.4%.

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Rapid Determination of the Relative Purity of Vitamin B_{12} (Cyanocobalamin) in Pharmaceutical Products

By C. F. BRUENING† and O. L. KLINE

The validity of the spectrophotometric procedure for determining the purity of cyanocobalamin in pharmaceutical products offered for injection is further demonstrated. This procedure is based on the ratio of absorptivities at 341 and 376 mμ. Samples of cyanocobalamin injection which met U. S. P. XVI specifications for purity and which were authenticated by inspectional evidence obtained by the Food and Drug Administration served as the reference standards.

In a previous report (1) a rapid spectrophotometric method was proposed to determine the relative purity of cyanocobalamin in pharmaceutical products involving the use of the ratio of absorptivities at 341 and 376 mμ. The validity of the method when applied to solutions containing only cyanocobalamin was established by using reference samples of cyanocobalamin U. S. P. (2).

With commercial preparations such as cyanocobalamin injection U. S. P. where authentic injections were unavailable, the validity of the method was established by use of the purity index. This index, by definition, is the ratio on a percentage basis obtained by dividing the cyanocobalamin content determined by the radioisotope tracer method (3) by the cyanocobalamin content determined by the U. S. P spectrophotometric method (2). Arbitrarily, an injection giving an index of 95% or more denoted that the cyanocobalamin used was of U. S. P. quality and that the injection was likewise of U. S. P. quality. It is the purpose of this report to establish the validity of the spectrophotometric method for commercial cyanocobalamin injections with greater certainty than that previously demonstrated in which the somewhat arbitrary purity index reference was used. The reference solutions used in this report consisted of cyanocobalamin injections which were of U. S. P. quality since they met all the requirements of the U. S. P. XVI, including in particular the test for cyanocobalamin solids, and were authentic as demonstrated by inspectional evidence.

To ascertain that samples of cyanocobalamin injection U. S. P. are made from cyanocobalamin U. S. P., the U. S. P. XVI (2) now contains under cyanocobalamin injection a test for "Limit of Cyanocobalamin Solids." Essentially this test determines the purity of the cyanocobalamin used in the injection. Specifications allow an upper limit of 1.2 mg. cyanocobalamin solids per mg. of cyanocobalamin. Solids in excess of this limit would indicate that impure cyanocobalamin had been used as an ingredient.

It might be pointed out, however, that this official solids test requires long periods of drying of the total solids and a gravimetric chloride determination which delays results for possibly two to three working days. On the other hand, the spectrophotometric method normally requires less than one hour and, because of its rapidity, we have used it as a sorting procedure in the Nutrition Division of the Food and Drug Administration. As previously reported, the proposed spectrophotometric method for cyanocobalamin injections established the ratio of absorptivities at 341 and 376 mμ of not more than 1.02 for injections of U. S. P. quality. Ratios above 1.02 would indicate that the product in question contained cyanocobalamin of less than U. S. P. XVI purity.

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EXPERIMENTAL

Eleven commercially prepared products of cyanocobalamin injection U. S. P. containing 1,000 mcg. cyanocobalamin per ml. and representing 11 manufacturers were selected as samples. The authenticity of each product was established through inspection by the Food and Drug Administration. After each sample was collected from an interstate shipment, a factory inspection was made to ascertain in particular that cyanocobalamin U. S. P. had been used. These samples were examined for cyanocobalamin solids by the method in U. S. P. XVI and the results are given in Table I. Since none of the observed values exceed 1.20 mg. cyanocobalamin solids per mg. of cyanocobalamin it can be concluded that, as measured by this test, all samples were of U. S. P. quality and were made from cyanocobalamin U. S. P.

Table I also shows the ratio of absorptivities at 341 and 376 mμ for the authentic samples determined as described in the previous report (1). Since all ratios are less than the prescribed maximum of 1.02, the conclusion can be drawn that all injections were made from cyanocobalamin of U. S. P. quality; and that the injections are also of U. S. P. quality. These data are thus in conformity with the data from the solids test demonstrating the validity of the ratio test.

Table I.—Cyanocobalamin Solids and Absorptivity Ratios of Cyanocobalamin Injections U. S. P.

Sample	Purity Index	Cyanocobalamin Solids, mg./mg. Cyanocobalamin	Absorptivity Ratio 341 mμ/376 mμ
1	100.5	1.10, 1.06	0.985
2	97.4	1.11 ..	0.989
3	98.7	1.13 ..	0.986
4	103.7	1.09 ..	0.985
5	...	1.10 ..	1.014
6	...	1.10 ..	1.000
7	...	1.13 ..	0.990
8	...	1.16[a]	1.013
9	...	1.08 ..	0.989
10	...	1.11, 1.12	0.984
11	...	1.08, 1.14	0.985

[a] Declared esters of *para*-hydroxybenzoic acid in this injection were removed as directed by the U. S. P. XVI (2) before proceeding with solids determination.

Table II.—Cyanocobalamin Solids and Absorptivity Ratios of Cyanocobalamin Injections Made from Impure Cyanocobalamin

Sample	Purity Index	Cyanocobalamin Solids, mg./mg. Cyanocobalamin		Absorptivity Ratio 341 mμ/376 mμ
1	96.9	1.42,	1.46	1.063
2	91.3	1.81,	1.83	1.085
3	91.8	3.07,	3.08	1.139
4		2.78,	2.81	1.170
5		1.39,	1.33	1.036
6		1.39,	1.41	1.047
7		1.37,	1.38	1.061
8		1.41		1.051
9		1.36,	1.36	1.040

The purity index values shown in Table I (and also those in Table II) were obtained as described in the previous report (1). All four values shown in Table I are in excess of 95%, indicating, by definition, that all the injections involved were of U. S. P. quality, a conclusion which is in conformity with that obtained from the two other tests shown in Table I.

It is interesting to note that if the authenticity of the samples as determined by inspectional evidence is accepted as valid, it appears that the data recorded here for cyanocobalamin solids for all the samples of Table I support the 1.20 limit as a reasonable selection.

Table II lists nine samples of commercially prepared injections of cyanocobalamin which were sampled while in interstate commerce and represent the output of four manufacturers. Although the products were represented to be of U. S. P. quality, inspectional evidence obtained by the Food and Drug Administration indicated that all injections were made from impure cyanocobalamin sources such as cobalamin concentrate N. F. (4) or similar products. The data shown for cyanocobalamin solids all exceed 1.20 mg./mg. cyanocobalamin, indicating that the injections were not of U. S. P. quality and could not have been made from cyanocobalamin U. S. P. The ratios of absorptivities at 341 and 376 mμ also exceeded 1.02 in all samples, permitting one to draw the same conclusion as that obtained from the solids test. Thus, with injections that do not conform to U. S. P. XVI requirements the ratio data parallel the cyanocobalamin solids data to a remarkable degree.

The purity index of sample No. 1 of Table II indicates by definition that this injection is of U. S. P. quality, a conclusion different from that obtained from the results of the other two tests of Table II. It is believed that the singular composition of this injection was responsible for the variance in conclusions because the corresponding purity index values were 87.2 and 86.2% when cyanocobalamin was determined by a counter current distribution method (5) rather than by the tracer method, indicating the presence of impure cyanocobalamin in the injection.

CONCLUSION

A study has been made of 20 cyanocobalamin injection samples consisting of 11 authentic samples of U. S. P. quality and nine samples of lower quality.

For each of the 20 samples the ratio of absorptivity at 341 and 376 mμ indicated, with approximately the same degree of certainty as did the U. S. P. XVI cyanocobalamin solids test, whether or not the injection was of U. S. P. quality and whether or not cyanocobalamin U. S. P. had been used in preparing the injection. This agreement of the two methods further establishes the validity of the spectrophotometric ratio method.

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Book Notices

Biochemical Preparations. Vol. 8. Edited by Alton Meister. John Wiley & Sons, Inc., 440 Park Avenue South, New York 16, N. Y., 1961. ix + 146 pp. 15 × 22.5 cm. Price $6.25.

The preparations included in this volume of the series are: Adenosine 5′-triphosphate, Toluenesulfonyl isopropylidene adenosine; S-Adensoyl-L-homocysteine; L-alanyl adenylate; L-methionyl adenylate; Amethopterin; Crystalline L-amino acid oxidase; α-Amylase from human saliva; α-Amylase from *Bacillus subtilis;* Diethylaminoethylcellulose; ECTEOLA-cellulose; Carboxymethylcellulose; Phosphorylated cellulose; 2-Deoxy-D-ribose; Hyamine hydroxide; δ-Hydroxy-L-lysine; Hydroxy-L-lysine, hydroxy-D-lysine, allohydroxy-L-lysine, and allohydroxy-D-lysine; Peptide chains (A and B) from beef insulin; L(+)-Lactic acid; DL-Lysine-1-C^{14}; DL-Lysine-6-C^{14} and DL-α-aminoadipic acid-6-C^{14}; β-Methyl-L-aspartic acid; β-Methyl-DL-aspartic acid; L- and D-threo-β-Methylaspartic acid; *p*-Nitrophenyl thymidine 5′-phosphate; Acetone-dried powder of beef pancreas; Spleen phosphodies-terase; 5:Phosphoryl α-D-ribofuranose 1-pyrophosphate (lithium salt); L-Proline; Pseudouridylic acid and pseudouridine; Sphingomyelin; Uridine 5′-diphosphate glucose; Uridine 5′-phosphate.

A Medical Greek Workbook. By James A. McCulloch. Duquesne University, Pittsburgh 19, Pa., 1959. 90 pp. 21.5 × 28 cm. Paperbound. Price $1.95.

A Medical Latin Workbook. By James A. McCulloch. Duquesne University, Pittsburgh 19, Pa., 1959. 93 pp. 21.5 × 28 cm. Paperbound. Price $2.

These two manuals are designed to furnish information relating to the origin of words and increasing the preprofessional student's vocabulary. No previous knowledge of Greek or Latin is prerequisite to their use.

Contents continued from back cover

JOSEPH LEVINE 506 Analysis of Acetylsalicylic Acid and Acetylsalicylic Acid Tablets

ELMER DE RITTER 510 Physicochemical and Biological Potency Relations for Isomerized Vitamin A Palmitate

Technical Articles

ALFRED N. MARTIN 513 Physical Chemical Approach to the Formulation of Pharmaceutical Suspensions

JOSEPH C. SAMYN 517 An Industrial Approach to Suspension Formulation

J. V. OLDSHUE 523 Mixing of Solid-Liquid Suspensions

KENNETH J. FREDERICK 531 Performance and Problems of Pharmaceutical Suspensions

Notes

JOHN J. HEFFERREN, CAROL DIETZ 535 Determination of Quaternary Ammonium Compounds

C. F. BRUENING, O. L. KLINE 537 Rapid Determination of the Relative Purity of Vitamin B_{12} (Cyanocobalamin) in Pharmaceutical Products

538 Book Notices

JOURNAL OF **Pharmaceutical Sciences**

June 1961 volume 50, number 6

Editorial

I In the Interest of Better Suspensions

Review Article

JONAS CAROL 451 Application of Infrared Spectrophotometry to Pharmaceutical Analysis

Research Articles

JOHN A. BILES 464 Optical Crystallographic Properties of Some Glucocorticoids

HERBERT A. BRAUN, LEHMAN M. LUSKY, STANLEY E. SRENSEK 469 Single-Injection Method for the Assay of Digitalis in Pigeons

LEMONT B. KIER 471 Triterpenes of *Poria obliqua*

MATHIAS P. MERTES, OLE GISVOLD 475 Ketals and Hemithioketals of 1-Methyl-4-piperidone and Tropinone

LEO GREENBERG 480 Comparative Laboratory Evaluation of Antiseborrheic Dermatitis Preparations

HORACE D. GRAHAM, LINNIE B. THOMAS 483 Interaction of Carrageenan and Other Hydrocolloids with Alkaloids I. Precipitation Studies

WILLIAM R. JONES, DONALD B. MEYERS, JOHN W. MARTIN 487 Isosteric Mercurial Diuretics

CLARK A. KELLY, MORRIS E. AUERBACH 490 Ion Exchange Separation and Colorimetric Determination of Phenylephrine in Pharmaceutical Products

PHILIP S. PORTOGHESE, LOUIS MALSPEIS 494 Relative Hydrolytic Rates of Certain Alkyl (b) *dl*-α-(2-Piperidyl)-phenylacetates

WILLIAM G. GROVES, HOWARD J. JENKINS 502 Investigation of the Cardiovascular Response of the Dog to 1-Phenyl-2-hydrazinopropane

HAROLD J. NICHOLAS 504 Isolation of a β-Sitosterol Complex from *Salvia sclarea*

Contents continued on inside back cover